CHEMICAL-ANALYTICAL AND SENSORY CHARACTERISATION OF KETTLE HOPPY AROMA OF BEER

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SAMENVATTING

Hop is een basisgrondstof voor bierproductie aangezien het aan de oorsprong ligt van de bittere smaak en het hoppig aroma van bier en op die manier een significante invloed heeft op de bierflavour. Het hoppig aroma van bier is een zeer controversieel onderzoeksonderwerp binnen de brouwerij-industrie. Het is nu algemeen aanvaard dat essentiële hopolie-afgeleide vluchtige verbindingen aan de basis liggen van het zeer gegeerde hoppig bieraroma. (Bio)chemische transformaties en verliezen tijdens het brouwproces veranderen het profiel van de hopoliecomponenten, wat resulteert in een hoppig aroma in bier dat duidelijk verschilt van het aroma van hop. Toch is de ware aard van het hoppig aroma tot op de dag van vandaag niet volledig opgehelderd. Vooral het nobele 'kettle hop' aroma, een typische flavourkarakteristiek van traditionele Pilsbieren dat verkregen wordt door het intensief koken van aromahopvariëteiten, blijft een actueel onderzoekstopic en kennis betreffende dit controversiële aspect van de bierflavour blijft contradictorisch en uiterst fragmentarisch.
Deze doctoraatstudie stelt het verkrijgen van wetenschappelijke inzichten betreffende de impact van het kookproces op analytische en sensorische karakteristieken van vluchtige hopoliecomponenten tot doel. Deze kennis is van primair belang om de chemische achtergrond van 'kettle hop' aroma te doorgronden.

In eerste instantie wordt een literatuuroverzicht gegeven over hop, het aroma van hop, (bio)chemische transformaties van vluchtige hopoliecomponenten doorheen het brouwproces en het hoppig aroma van bier.
Met het oog hop het verkrijgen van wetenschappelijke inzichten in veranderingen van het profiel van vluchtige hopoliecomponenten als gevolg van het kookproces, werden kookexperimenten uitgevoerd met totale essentiële hopolie (cv. Saaz) op laboschaal (in water). Headspace vaste fase micro-extractie gaschromatografie-massaspectrometrie (HS-SPME-GC-MS) analyse en multivariate data-analyse (Principale Componenten Analyse (PCA), Cluster Analyse (CA)) toonde duidelijk clustering aan tussen ongekookte en gekookte hopoliestalen. Dit werd toegeschreven aan een daling in de hoeveelheid terpeen koolwaterstoffen en een toename in de concentratie aan specifieke α-humuleen en β-caryofylleen-afgeleide oxidatieve- en hydrolyseproducten tijdens het kookproces. Ook werd er een reeks vluchtige verbindingen waargenomen, specifiek voor gekookte hopolie, wat wijst op *de novo* generatie van vluchtige verbindingen tijdens het kookproces. Sensorische evaluatie van gekookte hopolie in niet-gearomatiseerd bier, gebitterd met iso-α-zuren, toonde aan dat gekookte hopolie 'spicy/kruidige' impressies en 'kettle hop' aroma toekent aan bier.
Op basis van de interessante flavourkarakteristieken van gekookte hopolie, werd de zoektocht naar vluchtige hopolieconstituenten die betrokken zijn bij deze flavourimpressie

verder verfijnd door vaste fase extractie (SPE) fractionering van gekookte essentiële hopolie. Fracties die grote hoeveelheden aan geoxygeneerde componenten bevatten bleken 'spicy' en 'hoppy' effecten tot uiting te brengen na toevoeging aan water en bijgevolg werden deze fracties onderworpen aan grondige analytische karakterisering via HS-SPME-GC-MS. Vergelijking van de gekookte hopoliefracties met de corresponderende ongekookte hopoliefracties liet identificatie van vluchtige verbindingen die *de novo* worden gevormd tijdens het kookproces, toe. Sensorische evaluatie van ongekookte en gekookte hopoliefracties in niet-gearomatiseerd iso-α-zuur-gebitterd pilsbier toonde aan dat de fracties die grote hoeveelheden aan geoxygeneerde sesquiterpenoiden bevatten het meest relevant zijn met betrekking tot 'kettle hop' aroma en GC-olfactometrie (GC-O) duidde verschillende α-humuleen- en β-caryofylleen-afgeleide oxidatie- en hydrolyseproducten aan in geuractieve intervallen van deze fracties. Daarenboven werd een SPE methodologie ontwikkeld om geoxygeneerde sesquiterpenoiden uit commerciële pilsbieren aan te rijken. Na analytische profilering met HS-SPME-GC-MS konden iso-korajol, 4S-dihydrocaryofyllene-5-on, 6(5→4)-abeo-8,12-cyclo-caryofyllan-5-al en 6(5→4)-abeo-caryofyll-8(13)-en-5-al voor het eerst worden aangetoond in pilsbier. Ook werden vele componenten die *de novo* gevormd worden tijdens de laboschaal experimenten met essentiële hopolie (cv. Saaz) gedetecteerd in de commerciële bieren, wat erop wijst dat de deze experimenten relevant zijn voor de reële brouwerijpraktijk, en, GC-O analyse wees erop dat vele α-humuleen- en β-caryofylleen-afgeleide oxidatie- en hydrolyseproducten ook in flavouractieve intervallen van een commercieel bier elueerden.

Aanrijking van een sesquiterpeen koolwaterstof-fractie uit essentiële hopolie (cv. Saaz) en daaropvolgend koken (op laboschaal) van deze fractie bewees ondubbelzinnig oxidatie van sesquiterpeen koolwaterstoffen. SPE isolatie van de sesquiterpeen oxidatieproducten die *de novo* gevormd werden en toevoeging van deze fractie aan niet-gearomatiseerd iso-α-zuur-gebitterd pilsbier toonde een oorzaak-gevolg relatie aan tussen deze componenten en 'spicy/hoppy' aroma. Via GC-O analyse van deze oxidatieproducten-fractie werden twee intervallen met significante geuractiviteit vastgelegd. Humuleen epoxide III, humulenol II, caryofylla-4(12),8(13)-diene-5α/β-ol, (3Z)-caryofylla-3,8(13)-diene-5α-ol, 14-hydroxy-β-caryofylleen en (3Z)-caryofylla-3,8(13)-diene-5β-ol werden geïdentificeerd in deze intervallen en aangezien met uitzondering van humuleen epoxide III al deze oxidatieproducten ook geïdentificeerd werden in flavouractieve zones van een commercieel pilsbier, zijn deze vluchtige verbindingen belangrijke kandidaat impact-componenten voor 'kettle hop' aroma.

Door koken van toenemend hopolieconcentraties (cv. Saaz) in zowel water als wort kon een positieve correlatie aangetoond worden tussen de initiële hopolieconcentratie en de vorming van sesquiterpeen oxidatieproducten tijdens koken. Variëtale aspecten werden onderzocht via kookexperimenten (laboschaal) met essentiële hopolie cv. Saaz, cv. Hallertau Tradition, cv. Perle en cv. Magnum in wort. De resultaten tonen aan dat meeste *de novo*

gevormde sesquiterpeen oxidatieproducten identiek zijn voor de verschillende variëteiten. Kwalitatieve en kwantitatieve verschillen zijn te wijten aan de intrinsieke hopoliecompositie, die dus een belangrijke rol speelt in het potentieel van aromavariëteiten om 'kettle hop' aroma te ontwikkelen tijdens het brouwproces. Daarenboven konden selineen-afgeleide alcoholen en gerelateerde componenten (cadineen, muuroleen- en eudesmadiene-afgeleide alcoholen) niet gedetecteerd worden na koken van een selineen-rijke sesquiterpeen koolwaterstof-fractie (cv. Super Pride). Deze resultaten sluiten aan bij de hypothese dat deze componenten ontstaan uit biosynthese van de hopplant.

Finaal werden de wetenschappelijke inzichten die verkregen werden op laboschaal getoetst aan de reële brouwerijpraktijk door het brouwen van acht verschillende piloot-schaal pilsbieren, allen gearomatiseerd met Saaz hop pellets of essentiële hopolie (-afgeleide fracties). Analyse van wortstalen van een bier dat 'early kettle' hopping onderging wees op *de novo* vorming van sesquiterpeen oxidatieproducten tijdens het wortkoken, terwijl een toename in de concentratie van verschillende geoxygeneerde monoterpenoiden werd waargenomen na 'whirlpool' hopping. Hoewel vluchtige hopolieverbindingen die het brouwproces overleven en worden teruggevonden in het finale bier slechts in ppb-gehaltes worden gededecteerd, kwamen flavourverschillen als een resultaat van de verschillende hoppingpraktijken duidelijk tot uiting in de finale bieren. Combinatie van een 'early' en 'late' hop additie resulteerde in een zeer aangenaam bier met intens 'kettle hop' aroma, gekarakteriseerd door zowel 'spicy/kruidige' als 'florale/citrus-achtige' impressies. Hoewel het bier dat 'early kettle' hopping onderging ook deze 'spicy/kruidige' toetsen bevatte, bleek een delicaat evenwicht met een 'floraal/citrus-achtig' boeket noodzakelijk om het complete spectrum van 'kettle hop' aroma te verkrijgen. Additie van de sesquiterpeen oxidatieproducten-fractie na de fermentatie resulteerde in een bier dat de flavour van het 'early kettle' hopped bier benaderde. Via comprehensieve multidimensionale gas chromatografie gekoppeld aan massa spectrometrie (GCxGC-TOFMS) konden de eerder vooropgestelde kandidaat impactcomponenten voor 'kettle hop' aroma geïdentificeerd worden in zowel de fractie als het resulterende bier. Daarenboven bleek additie van deze oxidatieproductenfractie een positieve invloed te hebben op de waarneming van bitterheid en mondgevoelaspecten. Post-fermentatie aromatisering met gekookte hopolie resulteerde in een bier dat een hoge score ontving voor 'kettle hop' aroma en daarenboven was het flavourprofiel van dit bier zeer vergelijkbaar met een klassiek gehopt Pilsner-type bier. Er werd besloten dat deze twee nieuwe aromatiseringstechnologieën interesante flavourkarakteristieken toekennen aan bier en daarom veelbelovend technieken zijn om het 'kettle hop' aroma na te bootsen via post-fermentatie aromatisering.

SUMMARY

Hops constitute an indispensable raw material for beer production, since they significantly affect beer flavour by imparting a bitter taste and a fine hoppy aroma. Hoppy aroma of beer has been a matter of discussion amongst both brewers and researchers for decades. It is nowadays generally accepted that hop essential oil (-derived) volatiles are at the origin of this highly desired flavour characteristic. (Bio)chemical transformations and losses during the brewing process alter the hop oil volatile fingerprint, resulting in hoppy aroma in beer that is clearly different from the aroma of the hops. Nevertheless, the nature of hoppy aroma is up-to-date far from understood. Especially 'noble kettle hop' aroma, which is a typical flavour characteristic of traditional Pilsner-type beers obtained by vigorous boiling of aroma hops, has been a matter of debate and knowledge concerning this controversial issue remains contradictory and fragmentary.

This PhD study aims at providing scientific insights into the impact of the boiling process on the analytical and sensory characteristics of hop essential oil volatiles. Such knowledge is of utmost importance to completely understand the chemical background of 'kettle hop' aroma.

In first instance, a literature overview on hops, hop aroma, (bio)chemical transformations of hop oil volatiles during the brewing process and hoppy aroma of beer is presented.

In order to obtain scientific insights on changes in the hop oil volatile profile upon boiling, lab scale boiling experiments (in water) with total hop essential oil (cv. Saaz) were conducted. HS-SPME-GC-MS analysis and multivariate data analysis (Principal Component Analysis (PCA), Cluster Analysis (CA)) revealed clustering between unboiled and boiled hop essential oil samples, due to decreases in terpene hydrocarbon levels and an increase in the level of particular α-humulene and β-caryophyllene oxidation and hydrolysis products upon boiling. Also a series of volatiles specific for boiled hop oil could be observed, pointing to *de novo* generation of volatiles as a result of the boiling process. Sensory evaluations of boiled hop essential oil in non-aromatised iso-α-acid bittered lager beer demonstrated that boiled hop oil imparts 'spicy/herbal' impressions and 'kettle hop' aroma to beer.

Seen the interesting flavour characteristics (spicy, herbal notes) of boiled hop essential oil, the search for hop oil volatiles involved in this flavour impression was further focused by Solid Phase Extraction (SPE) fractionation of boiled hop essential oil. Fractions with high levels of oxygenated compounds appeared to express 'spicy' and 'hoppy' notes when spiked to water and were therefore subjected to comprehensive analytical profiling via HS-SPME-GC-MS. Comparison of the boiled hop oil fractions with the corresponding unboiled hop oil fractions allowed for identification of additional volatiles formed *de novo* during the boiling

process. Sensory evaluation of the unboiled and boiled hop oil fractions in non-aromatised iso-α-acid bittered lager beer suggested that fractions containing high oxygenated sesquiterpenoid levels are most relevant in relation to 'kettle hop' aroma and GC-olfactometry (GC-O) indicated several α-humulene and β-caryophyllene oxidation and hydrolysis products in flavour-active intervals of these fractions. Moreover, an SPE methodology was developed to enrich oxygenated sesquiterpenoids from commercial kettle hopped lager beers. Upon HS-SPME-GC-MS analytical profiling, iso-korajol, 4S-dihydrocaryophyllene-5-one, 6(5→4)-abeo-8,12-cyclo-caryophyllan-5-al and 6(5→4)-abeo-caryophyll-8(13)-en-5-al could be reported in lager beer for the first time. Moreover, many compounds formed *de novo* upon lab scale boiling of hop oil (cv. Saaz) were detected in the commercial beers, indicating that the lab scale boiling experiments are relevant for real brewing practice. Moreover, GC-O analysis of a commercial kettle hopped beer demonstrated that several α-humulene and β-caryophyllene oxidation products also elute in flavour-active zones.

Enrichment of a sesquiterpene hydrocarbon fraction from total hop essential oil (cv. Saaz) and subsequent lab scale boiling of this fraction unambiguously proved oxidation of sesquiterpene hydrocarbons. Moreover, SPE isolation of the sesquiterpene oxidation products formed *de novo* and addition of this fraction to non-aromatised iso-α-acid bittered lager beer demonstrated a cause-and-effect relationship between these constituents and 'spicy/kettle hop' flavour. Via GC-O analysis of the oxidation product fraction, two intervals with significant odour-activity were established. Humulene epoxide III, humulenol II, caryophylla-4(12),8(13)-diene-5α/β-ol, (3Z)-caryophylla-3,8(13)-diene-5α-ol, 14-hydroxy-β-caryophyllene and (3Z)-caryophylla-3,8(13)-diene-5β-ol were identified in these intervals and since except for humulene epoxide III all these oxidation products were also found in flavour-active zones of a commercial kettle hopped lager beer, these volatiles are candidate key impact compounds for 'kettle hop' aroma.

By boiling increasing hop essential oil (cv. Saaz) concentrations in both water and wort, a positive correlation between the initial hop oil concentration and formation of sesquiterpene oxidation products upon boiling was found. Varietal aspects were investigated by boiling experiments with hop essential oil cv. Saaz, cv. Hallertau Tradition, cv. Perle and cv. Magnum in wort, suggesting that most of the sesquiterpene oxidation products formed *de novo* during boiling are identical for the different varieties. Qualitative and quantitative differences may be attributed to the intrinsic hop oil composition, which may play a key role in the potential of hop varieties to develop 'kettle hop' aroma during the brewing process. Moreover, selinenols and related compounds (*i.e.* cadinols, muurolols, eudesmols) could not be detected upon boiling of a selinene-rich sesquiterpene hydrocarbon fraction cv. Super Pride, supporting the hypothesis that these compounds are biosynthesised by the hop plant.

Finally, scientific insights obtained on lab scale were verified in real brewing practice by brewing eight different pilot-scale lager beers, aromatised with Saaz hop pellets or hop oil (-derived fractions). Analysis of wort samples of an 'early' kettle hopped beer indicated *de novo* formation of sesquiterpene oxidation products during the wort boiling process, whereas increases in the level of several oxygenated monoterpenoids could be observed upon 'whirlpool' hopping. Although the amount of hop oil-derived volatiles surviving up to the final beer was at the low ppb level, flavour differences as a result of the different hopping practices clearly came to expression in the final beers. Combination of 'early' and 'late' kettle hopping resulted in a highly appreciated beer with intense 'kettle hop' flavour, characterised by both 'spicy/herbal' and 'floral/citrusy' connotations. Although an 'early' kettle hopped beer also expressed the characteristic 'spicy/herbal' note, it appeared that a delicate balance with a 'floral/citrus' bouquet is required to obtain the full complex spectrum of 'kettle hop' flavour.

Aromatisation by post-fermentation addition of the sesquiterpene oxidation product fraction resulted in a beer that approached the flavour of an 'early' kettle hopped beer.

Using comprehensive multidimensional gas chromatography-mass spectrometry (GCxGC-TOFMS), the previously proposed candidate key impact compounds could be identified in both the fraction and the resulting beer. Moreover, addition of this oxidation product fraction positively influenced the bitterness perception and significantly increased mouthfeel aspects. Post-fermentation aromatisation with boiled hop oil resulted in a beer that received a high score for 'kettle hop' aroma and, moreover, the flavour profile of this beer was highly comparable to a conventionally kettle hopped Pilsner-type lager beer. It was concluded that these two new aromatisation technologies impart interesting flavour characteristics to beer and are promising techniques to mimic 'kettle hop' flavour by post-fermentation aromatisation.

LIST OF ABBREVIATIONS AND SYMBOLS

°C	Degrees Celsius
°P	Degrees Plato; 1°P = 1 % (w/w) soluble extract
3S4MP	3-Sulfanyl-4-methylpentan-1-ol
3S4MPA	3-Sulfanyl-4-methylpentyl acetate
4MMP	4-Mercapto-4-methylpentan-2-one
a	Slope
AEDA	Aroma Extract Dilution Analysis
ANOVA	Analysis Of Variance
ASBC	American Society of Brewing Chemists
b	Intercept
B.C	Before Christ
C	Carbon atom
C18	Silica column with octadecyl groups
CA	Cluster Analysis
CHARM	Combined Hedonic Aroma Response Method
CO_2	Carbondioxide
CV	Coefficient of Variation
cv.	Cultivar
DA	Discriminant Analysis
DF	Detection Frequency
DMDS	Dimethyl disulfide
DMS	Dimethyl sulfide
DMTS	Dimethyl trisulfide
e.g.	*Exempli gratia*
EBC	European Brewery Convention
EDA	Exploratory Data Analysis
(E)FA	(Exploratory) Factor Analysis
EFBT	Lab for Enzyme, Fermentation and Brewing Technology
et al.	*Et alii*
EtOH	Ethanol, food grade
eV	Electron volt
FD	Dilution Factor
FID	Flame Ionisation Detection
FS	Full Scan
g	Gram
g/L	Gram per liter
GC	Gas Chromatography

GC-FID	Gas Chromatography - Flame Ionisation Detection
GC-MS	Gas Chromatography - Mass Spectrometry
GC-MS/MS	Gas Chromatography - Tandem Mass Spectrometry
GC-O	Gas Chromatography - Olfactometry
GC-O/MS	Gas Chromatography - Olfactometry / Mass Spectrometry
GCxGC	Multidimensional Gas Chromatography
GCxGC-TOFMS	Two-dimensional Gas Chromatography with Time-Of-Flight Mass Spectrometry
h	Hour
H/C	Humulene to caryophyllene ratio
hL	Hectoliter
HPLC	High Performance Liquid Chromatography
HS-SPME	Headspace Solid Phase Micro-Extraction
HS-SPME-GC-MS	Headspace Solid Phase Micro-Extraction in combination with Gas Chromatography and Mass Spectrometry
Hz	Hertz
i.d.	Internal diameter
i.e.	*Id est*
IBU	International Bitterness Unit
kg	Kilogram
LOD	Limit Of Detection
m/z	Mass to charge ratio
MBAA	Master Brewers Association of Americas
mg/L	Millligram per liter
min	Minutes
MLR	Multivariate Linear Regression
MQ	Milli-Q (purified water)
MS	Mass Spectrum
MS/MS	Tandem Mass Spectrometry
MSP	4-Methyl-4-sulfanylpentan-2-one
MW	Molecular Weight
n	Number of repititions
n°	Number
NIST	National Institute of Standards and Technology
OAV	Odour Activity Value
OGA	Olfactometry Global Analysis
OSs	Oxygenated sesquiterpenoids
PARC	Pattern Recognition

PC	Principal Component
PCA	Principal Component Analysis
PCR	Prinicpal Component Regression
PDMS	Polydimethylsiloxane
pH	Measurement for acidity
PHA	Pure Hop Aroma
PLS	Partial Least Squares
PLS-DA	Partial Least Squares Discriminant Analysis
ppb	Parts per billion
ppm	Parts per million
R	Coefficien of correlation
R^2	Coefficient of determination
RC	Reference Compound
RI	Retention Index
RMSEC	Root Mean Square Error of Calibration
RMSECV	Root Means Square Error of Cross Validation
rpm	Rotations per minute
RTX-1	Non-polar polydimethylsiloxane chromatography column
s	Seconds
S.D.	Standard Deviation
S/N	Signal to noise ratio
SFE	Supercritical Fluid Extraction
SHCs	Sesquiterpene hydrocarbons
SIM	Selected Ion Monitoring
SIMCA	Soft Independent Modeling of Class Analogies
SOPs	Sesquiterpene oxidation (incl. rearrangement and hydrolysis) products
SPE	Solid Phase Extraction
SPME	Solid Phase Micro-Extraction
Spp.	Species
T90	Type 90; 100 kg hop cones results in 90 kg pellets
UK	United Kingdom
USA	United States of America
v/v	Volume ratio
v/v%	Percent volume by volume, mL per 100 mL
v/w%	Percent volume by weight, mL per 100 gram
VIP	Variable Importance in Projection
w/w	Weight ratio
w/w%	Percent weight by weight, mass (g) per 100 gram

TABLE OF CONTENTS

GENERAL INTRODUCTION AND SCOPE

Outline of the research subject, general aim of the PhD project and overview of chapters.

GENERAL INTRODUCTION AND SCOPE

The flavour that characterises a certain beer is an essential criterion as regards the quality of the product. The analytical and sensorial complexity of the flavour of beer can be attributed to a diversity of components that can lead to different impressions of flavour. Even though hops are only added in relatively small doses during brewing, this raw material has a very large impact on the sensory aspects of the beer flavour[1,2]. Particularly the hop α-acids and the essential oil of the hops respectively impart the bitterness and the desired "hoppy" aroma[3]. The underlying chemistry of the bitterness is well-known so the desired bitterness intensity can be specified in an accurate way. However, because of the very complex chemical composition of the hop oil as such, the insufficient knowledge of the behaviour of the hop oil components during the brewing process (cf. many possible (bio-) chemical conversions[4]) and mostly inadequate insights in the nature of the flavour-active volatile components, the chemical background of the hoppy aroma of beer is still ill-defined[5,6]. The aroma of hops is clearly different from the resulting hoppy aroma in the final beer and moreover, the hoppy aroma can be attributed to the complex interactions (additive/synergetic) between the various aroma active components in the beer matrix[7,8].

For a very long time, the hop-research was particularly focussed on the identification of unknown volatile components[4]. Guadagni *et al.*[9] published a systematic study on the contribution of individual volatile hop oil components to the aroma of beer for the first time. After many years of research with the aid of gas chromatographic and olfactometric techniques, a lot of those hop components were associated with certain impressions of the hoppy aroma of beer, which however not always implies a cause-effect relationship between the presence of this component and the investigated aroma impression. Either way, monoterpene alcohols like for instance linalool, entail a floral top note[10–15]. On the contrary, the oxygenated sesquiterpenoids (*e.g.* humuladienone, humulene epoxides) are related to the spicy aspect of the hoppy aroma, despite the fact that the identified, individual sesquiterpenoids don't manifest the spicy aroma and also appear below their flavour threshold value in beer[5,10,16–20]. Conclusively, water-soluble hop glycosides were indicated as possible precursors of hop aromas which would contribute to the beer flavour[21–23], but also in this case there is still quite some additional research required.

In practice, in the case of conventional hopping (worldwide still approx. 80% of total hops usage[24]), the aromatisation is usually realised by addition of hops near the end of the boiling process ('late kettle'-hopping). To a lesser extent brewers also use the technology of 'early kettle' hopping, thereby adding relatively expensive European aroma hops at the onset of wort boiling. This particular hopping practice would impart 'noble kettle hop' aroma to their

beers, which is regarded a subtle yet refined and highly desired flavour characteristic of classic Pilsner-type lager beers and is defined by an unique 'spicy/herbal' bouquet[25–28]. Most breweries have been applying this conventional hop aromatisation in a purely empirical way, without in fact having scientifically based insights into the relation between the applied hop technology and the flavour characteristics of 'kettle hop' aroma in the final beer.

Therefore, this PhD project embraces a detailed analytical and sensorial characterisation of the complex, conventional 'kettle hop' aroma of beer. In general, the aim was to acquire fundamental insights in the relation between the aroma of hops as such and the mix of hop derivative flavour-active volatile compounds, whether or not being formed *de novo* during the boiling processs, and, to investigate the practical relevance ot the research results by preparation of specifically hopped test brews on a pilot scale.

First, in **Chapter 1**, an extensive literature study on hops, hop aroma, (bio)chemical transformations of hop oil volatiles and hoppy aroma in beer is presented, which forms the scientific basis for the experimental work (**Chapter 2**-**6**).

In **Chapter 2**, the impact of the boiling process on the analytical fingerprint of hop oil-derived volatiles was investigated by lab scale boiling of hop essential oil (cv. Saaz) in water and subsequent HS-SPME-GC-MS analysis. Chromatograms of unboiled and boiled hop essential oil were compared to deduce information on the behaviour of general hop oil compound classes upon boiling and volatiles formed *de novo* were pinpointed. The impact of the boiling process on flavour characteristics of hop essential oil was evaluated by sensory analysis of boiled total hop essential oil in non-aromatised iso-α-acid bittered lager beer.

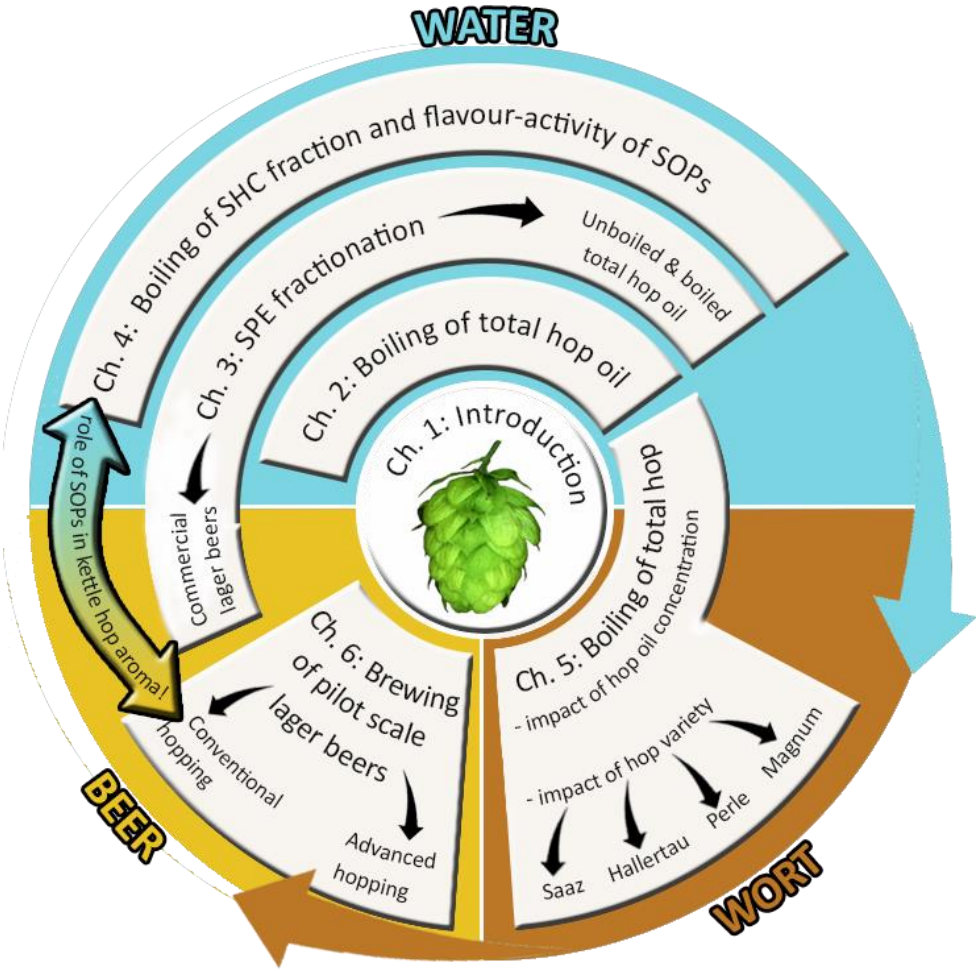
Since hop oil is an enourmously complex mixture comprising over 1000 different volatiles, co-elution of volatiles in the chromatograms impedes profound analytical characterisation of unboiled and boiled hop oil and, moreover, flavour characteristics are difficult to relate to particular chemical compound classes. Therefore, in **Chapter 3**, Solid Phase Extraction (SPE) was used to fractionate unboiled and boiled hop oil. These fractions were subjected to comprehensive analytical fingerprinting and sensory evaluation. Individual flavour-active volatiles were determined using GC-olfactometry (GC-O), whereas the flavour characteristics of the total fractions were evaluated in beer. Moreover, an SPE method was developed to enrich spicy compounds from commercial kettle hopped lagers, whereupon the resulting fraction was analytically characterised and screened for flavour-active compounds via GC-O.

Since the results of **Chapter 2** and **3** pointed to *de novo* formation of various oxygenated sesquiterpenoids upon boiling, we focussed on these compounds in **Chapter 4**. Sesquiterpene hydrocarbons (SHCs) were isolated from total hop essential oil (cv. Saaz) using SPE, whereupon the enriched SHC fraction was boiled (lab scale, in water). The resulting sesquiterpene oxidation products (SOPs) were subsequently isolated via SPE and the

resulting SOP fraction was evaluated by sensory analysis in beer. Since the fraction expressed interesting flavour characteristics related to 'kettle hop' aroma, GC-O was performed to determine flavour-active volatiles in order to propose key impact flavour compounds for 'kettle hop' aroma.

Chapter 5 elaborates on the the impact of the hop oil concentration on changes in the hop oil volatile profile upon boiling in water and wort. Moreover, the varietal aspect is investigated by comparison of *de novo* formation of volatiles upon boiling of hop essential oil cv. Saaz, cv. Magnum, cv. Halletertau Tradition and cv. Perle.

Finally, in **Chapter 6**, the relevance of scientific insights obtained in the previous chapters for real brewing practice is explored by brewing of different lager beers, thereby varying the time point of hop addition. Moreover, the flavour potential of unboiled hop essential oil, boiled hop essential oil and the SOP fraction (obtained in **Chapter 4**) for brewing practice was investigated by post-fermentation addition and sensory evaluation of the resulting beers. Comprehensive multidimensional gas chromatography (GCxGC-TOFMS) was employed to screen the beer aromatised with the oxidation product fraction for the candidate key impact flavour compounds proposed in **Chapter 4**.



Schematic overview of coherence between different chapters.

Chapter 1

INTRODUCTION

This chapter gives an overview on hops, the aroma of unprocessed hops, chemical oxidations and biotransformations of hop oil volatiles by yeast during the brewing process and hoppy aroma in beer.

Contributions

The writing and literature study was performed by Tatiana Praet. The final manuscript was revised and adapted after critical input by Prof. Luc De Cooman and Dr. Filip Van Opstaele.

1 INTRODUCTION

1.1 The brewing process

The word *beer* comes from the Latin word *Bibere* (to drink). Beer is an alcoholic beverage, which contains relatively low levels of alcohol (international averages range from 3.9 v/v% to 5.1 v/v%) when comparing to most other alcoholic beverages[29]. Production of this aqueous drink is based on fermentation of sugars derived from starch and flavouring by hops[30]. Yeast (*Saccharomyces* spp.) is able to ferment sugar to ethanol according to the following reaction: $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$. If the sugars are derived from grapes, the end product is *wine*. When sugars are derived from apples, the end product will be hard *cider*. If the sugars are from grain, the end product is *beer*[29]. Records of brewing beer date back to 7000 B.C. in Babylon[2], making it one of the oldest beverages produced by humans. Nowadays, beer is world's favourite alcoholic beverage and world-wide beer production exceeds 1.9 billion hL per year. Clearly, world-wide beer production keeps increasing as the beer production a decade ago was estimated at 1.6 billion hL per year[31]. From the actual total beer production, 704, 572 and 523 million hL are produced in Asia, America, and Europe, respectively. Belgian beer production is estimated at 18 million hL, ranking it on the 24th place amongst countries in Europe with the largest beer production[31]. The above data demonstrate the enormous and increasing popularity of beer.

In general, the brewing process consists of mashing, wort boiling and fermentation (see **Figure 1-1**) and, basically, four essential ingredients are necessarily used in the intricate beer brewing process, *i.e.* water, malt, yeast and hops. Barley malt provides the body of beer and, mostly, a few hundreds of grams are used for one litre of beer[30]. Barley malt is obtained by a controlled germination of barley, during which enzymes (amylases, proteases) are formed which will be essential to degrade starch and proteins. Although barley malt may be partly substituted by other starch-rich ingredients (*e.g.* rice, corn, wheat, sorghum), barley remains the predominant grain used in the majority of the world's beers[29,30]. Nevertheless, adjuncts such as cereals, syrups and sugars are frequently used to supplement malt starch. Typical examples are flaked cereals, flours (usually made from wheat) and grits, which contain the coarse starchy endosperm particles of *e.g*. corn or rice. Syrups are produced by acid/enzyme treatments of starch from corn, barley or wheat, whereas sugars usually consist of cane sucrose[32]. During mashing, milled malt is mixed with brewing water and heated to specific temperatures (including 63°C and 72°C), which will activate the malt enzymes, leading to a mixture of sugars and peptides or amino acids[30]. After mashing, the aqueous mixture is filtered to produce sweet wort, which contains the soluble and suspended substances

derived from the malt. Recovering wort from the residual grains is perhaps the most skilled part of brewing since many brewers aim at recovering a bright wort (not containing too many insoluble particles) with as much extract as possible[29]. After filtration, wort is transferred to the brewing kettle, where it is boiled for at least one hour with addition of hops (*Humulus lupulus* L.). Although hops are added in small quantities and the hopping accounts for less than 1% of the price of beer, it has a disproportionate effect on product quality[29]. Besides imparting hoppy aroma, the most important asset of hops is the resulting bitter taste, although hops also play a positive role in wort clarification and bacteriological as well as foam stability of beer[30]. Besides isomerisation of hop α-acids into the bittering iso-α-acids, wort boiling serves various other functions such as inactivation of residual enzymes, sterilisation, removal of unwanted volatiles, precipitation of protein/polyphenol complexes (as 'hot break' or 'trub'), colour formation through Maillard reactions and concentration of the wort because of evaporation. After wort boiling, insoluble materials and spent hops are removed during wort clarification and the hopped wort is cooled to the fermentation temperature. At the cooling stage, 'cold break' is formed and air or oxygen is introduced to promote aerobic growth of the pitched yeast[29]. During the anaerobic phase, yeast cells convert fermentable sugars into ethanol and carbon dioxide. *Saccharomyces cerevisiae* yeast strains settle to the surface of fermenting wort and are used to produce ale beers, whereas *Saccharomyces pastorianus* strains are typically used to produce lager beer and settle to the bottom of the fermentation tank. 'Top' and 'bottom' fermentations are usually completed within a few days at temperatures as high as 20°C and up to several weeks at temperatures as low as 6°C, respectively[29]. Lager yeasts produce up to 5 v/v% ethanol, whereas ale yeast strains operate at ambient temperature and are resistant to ethanol concentrations up to 12 v/v%. After primary fermentation, a maturation or lagering period of several weeks at 0°C usually follows to convert unwanted compounds such as diacetyl and pentane-2,3-dione. Once concentrations of such compounds have sufficiently decreased, beer can be filtered, adjusted to the required carbonation and packaged into kegs, bottles or cans. Pasteurisation may be used to obtain a prolonged conservation of beer[30].

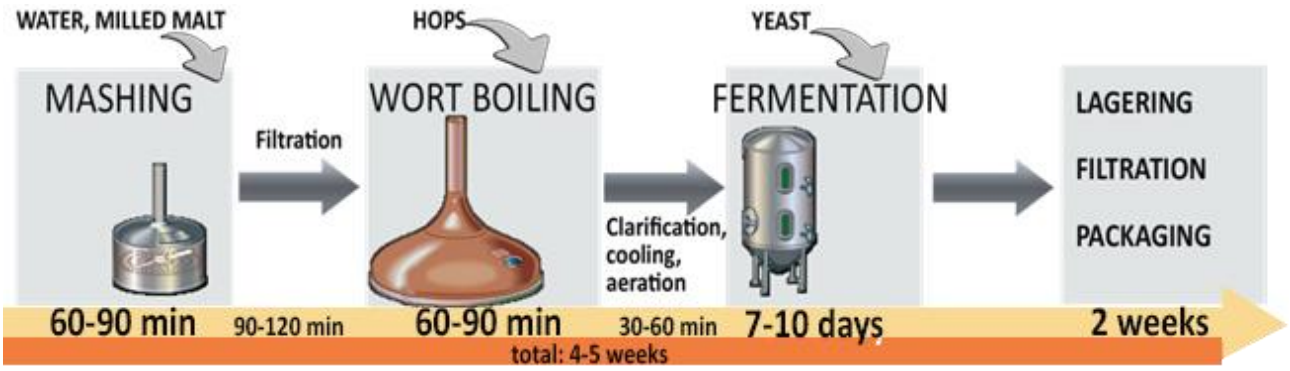


Figure 1-1. Schematic overview of lager beer production.

Various beer types exist and beers can be classified according to overall style, strength, colour, principal grist ingredient, region of original production, and applied production technologies. Regarding the beer style, beers have traditionally been categorised in *ale beers*, fermented from relatively dark well-modified highly kilned malts using top fermenting yeast strains and relatively high fermentation temperatures (10-20°C), and *lager beers*, fermented from less modified, gently kilned lightly coloured malts using bottom fermentation at lower temperatures (0-10°C). Nowadays, boundaries between these two beer catergories start to blur since, for example, lager strains may be used to produce ale beers and vice versa. Beers may also be classified in terms of original extract or gravity of the worts at the start of the fermentation. Another way to divide beers is on the basis of colour. For example, ales are traditionally classified into pale ales, brown ales, porters and stouts and although most lagers are pale, particular lagers such as Dunkel and Schwarzbier are dark. Beers may also be divided based on the principal grist compound used. For example, Weissbier is made from malted wheat, whereas many beers in Africa are made from sorghum. Pils beer or Pilsner was traditionally brewed in Pilsen (Czech Republic), demonstrating that beers can be classified according to their region of original production. However, nowadays, Pilsner beers lost their strict definition and have become a synonym for mid-strength lagers. Finally, beers are classified according to the technology used. Light beers are perhaps the most obvious example of this[29].

1.2 Beer flavour

Flavour, appearance and consistency and texture are the main attributes of a food or beverage item. In terms of perception, most or all of these attributes overlap[33]. Flavour has been defined as the sum of perceptions resulting from stimulation of the sense ends that are grouped together at the entrance of the alimentary and respiratory tracts[34]. For practical sensory analysis, Caul[35] restricted this term to the impressions perceived from a product in the mouth, defining flavour as the aromatics (olfactory perceptions caused by volatile substances released from a product in the mouth via the posterior nares), the tastes (gustatory perceptions caused by soluble substances in the mouth) and the chemical feeling factors (which stimulate nerve ends in the soft membranes of the buccal and nasal cavities), such as astringency, spice heat, cooling, bite, metallic flavour and umami taste[33]. Thus, in other words, the term flavour covers both odour and aroma, taste and mouthfeel. 'Odour' is the perception of volatiles by the olfactory mucous membrane in the nasal cavity when a food or beverage is sniffed. When the food/beverage is taken into the mouth, chewed and swallowed, a portion of the volatiles in the mouth pass via the nasopharyngeal passage into the nose where they contact the olfactory epithelium and are perceived as 'aroma'. Gustation or taste involves the detection of stimuli dissolved in water, oil, or saliva by the

taste buds that are primarily located on the surface of the tongue as well as in the mucosa of the palate and areas of the throat[33]. The primary taste attributes are salty, sweet, sour and bitter and, recently, umami and fatty were also classified under taste perceptions[36]. The perception of a food product or beverage by the trigeminal nerve ends of the surface of the oral cavity is described by the term 'mouthfeel'[33,37]. Chemical irritants that trigger such perceptions of burn, heat, cold and pungency are, for example, ammonia, onion and chili peppers[33]. Responses to mild irritants such as carbonation and the heat of peppers and other spices may contribute to the acceptance of a product[38].

The complexity of beer flavour is demonstrated by the flavour wheel of Meilgaard and Peppard[39] (see **Figure 1-2)**, which is a system of flavour terminology in which each separately identifiable flavour note in beer has a name. The original flavour wheel, developed by Meilgaard *et al.*[40], was the result of joint working groups of the American Society of Brewing Chemists (ASBC), the European Brewery Convention (EBC) and the Master Brewers Association of Americas (MBAA).

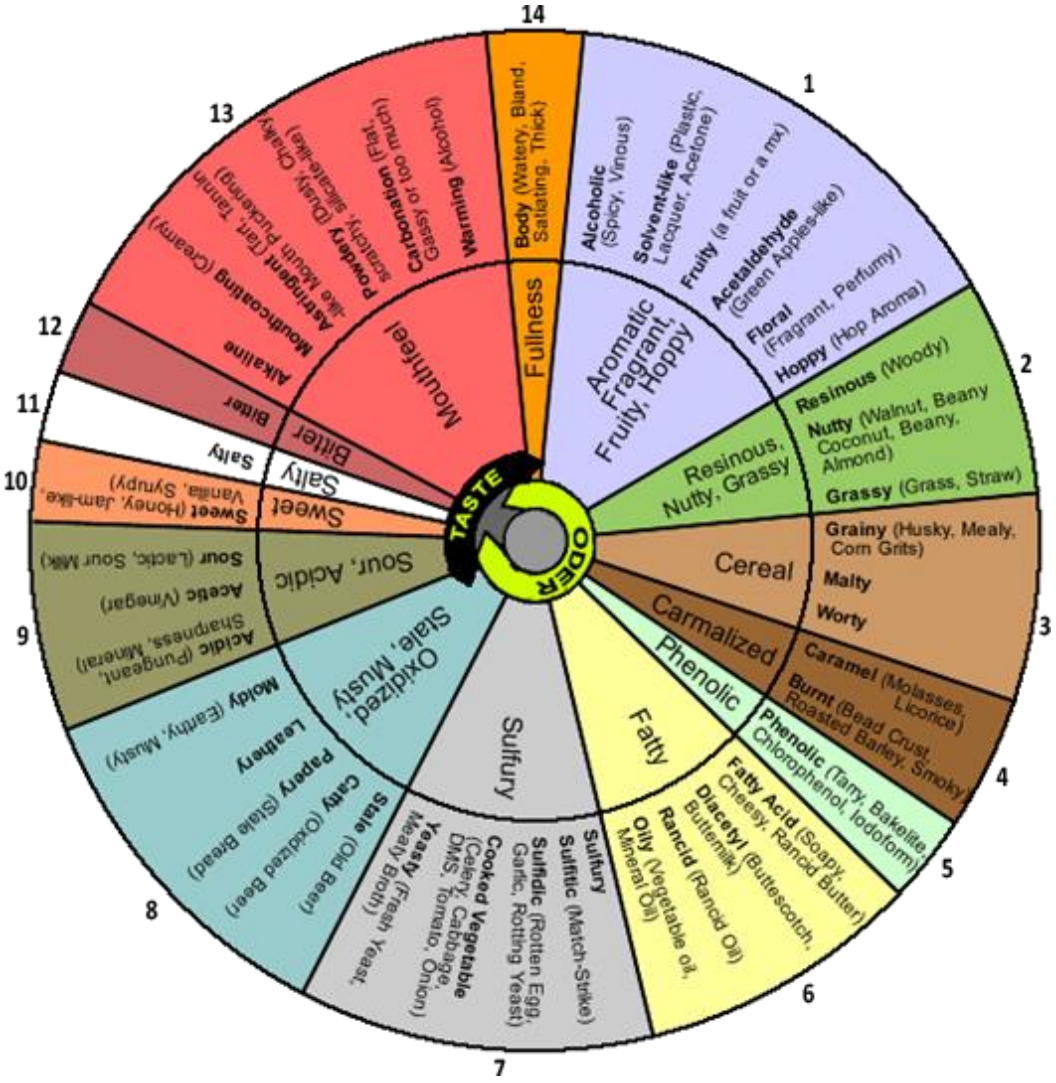


Figure 1-2. The beer flavour wheel, showing class terms and first tier terms[39].

The system, which has 44 principal terms and 78 second-tier terms, divides the odour into eight classes in an approximate order from pleasant (class 1: aromatic, fragrant, fruity, floral) to unpleasant (class 8: oxidised, stale, musty). The taste of beer is described in terms of the four basic tastes (class 9-12), mouthfeel (class 13) and fullness (class 14).

Over the past years, the beer flavour wheel has gained general acceptance. Nevertheless, the terminology remains a matter of debate and expansions[37] and variations[36] of the original beer flavour wheel have been proposed. Langstaff and coworkers[37] introduced an additional segment, placing mouthfeel next to odour/aroma and taste. This expansion subdivides the mouthfeel aspect into carbonation, fullness and afterfeel. Schmelze[36] proposed a revised beer flavour wheel, structuring terminology according to sensory standards. This beer flavour wheel should be easier to apply by people not familiar with the complex beer flavour chemistry.

The predominant influences on overall beer flavour are derived from hop bitterness and aroma, malt components, yeast metabolism products, and adjuncts. For example, the malt-derived component DMS has received much attention and imparts a cabbage/vegetable aroma, which can be perceived in some European lagers. Because of its high concentration, ethanol makes a major contribution to beer flavour, although the minor yeast metabolism products give beer its characteristic flavour[32]. Most of the constituents of beer are present at levels just below those at which they are easily perceived[32]. Because both the concentrations and flavour thresholds of compounds can vary widely, the term 'flavour unit' (FU) was introduced, which is the ratio of the concentration of a flavour-active compound and its threshold value[41]. However, synergistic and antagonistic interactions between hop, malt and yeast metabolism constituents give the specific flavours associated with beer[32]. In general, the alcoholic note, carbonation mouthfeel by CO_2 and hop-derived bitterness are considered as the primary beer flavour attributes[42].

Although large amounts of malt (typically 150 g/L) are used in the production of beer compared to the amounts of hops (typically 1 g/L), hops have a major impact on overall beer flavour. In particular the hop lupulin fraction is by far the most important fraction, since it contains the α-acids from which are derived the principal bittering substances of beer, and the essential oil, which is at the origin of hoppy aroma of beer[39]. According to Verzele[43], tasting of unhopped beer is "a most revealing experience", as malt liquor is very sweet and the malty flavour is not really pleasant. In order to balance this sweetness, hop bitterness is of utmost importance[2]. Obviously, hops have a high impact on the beer flavour and, consequently, understanding the rather complex hop chemistry is of major importance for brewers and hop scientists.

1.3 Hops

1.3.1 Introduction

The brewing of beer dates back to 7000 B.C in Babylon[2]. The first record of the use of hops for brewing dates from 1079[2]. In the 12th century, the value of hops for flavour and preservation of alcoholic beverages appears to have been recognised and from the 13th century, hops began to replace gruit as a flavouring for beer in Germany[2]. In 1516, hops became the sole flavouring ingredient of beer and this unique status was codified in the Bavarian Reinheitsgebot which evolved into the modern Purity Law for beer brewed in Germany[44].

There are three species of hops: *Humulus lupulus* L., *Humulus japonicus* and *Humulus yunnanensis* Hu. Whereas *Humulus japonicus* contains no resin and is merely ornamental, H*. lupulus* is rich in resins and oils, the sources of beer bitterness and hoppy aroma[45]. The hop genus is within the family *Cannabinaceae* and a close relative of the hop is *Cannabis sativa*, also known as Indian hemp or marijuana[29]. Hops (*Humulus lupulus* L.) is a perennial, dioecious (*i.e.* separate male and female plants) climbing plant[46]. In general, only the female plants are cultivated since they bear the inflorescenses which, because of their shape, are called hop cones[47].

The components of hops with brewing value (*i.e.* the resins and the oils) are located in the cones of female plants[29]. The lupulin glands (see **Figure 1-3**), which are located at the base of the bracteoles, contain the resins and oils[29,32]. Lupulin glands develop as the hop ripens. These beaker-like glands contain lupulin, a yellow sticky powder consisting of secondary metabolites, secreted by the hop plant. The gland is covered by a membrane to prevent its contents from escaping. The lupulin content of the hop cone can reach up to 32% (w/w)[39].

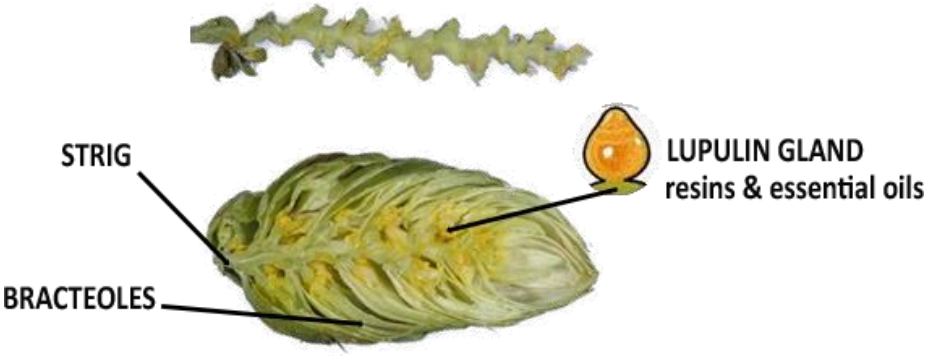


Figure 1-3. Hop cone and its parts.

Hop growth takes place between April and July (in the Northern hemisphere) and is vigorous and fast[1]. During July and August, the flowers of the female hop plant develop to form hop cones. Once these cones have reached ripening, the crop is harvested using picking

machines[1]. The harvest time, usually from the end of August until the end of September, has a major impact on the hop quality and depends mainly on hop variety and weather conditions[48,49]. Daily determination of the α-acid content of the cones is of utmost importance to make a decision regarding the right time for picking. When hops are picked before their technological ripeness, the smell of the hop cones will be fresh but levels of both hop acids and essential oil will be too low[46,50]. In order to harvest the hop plants, the bines are cut down, transporting them to the picking machine which strips the cones from the bines. Next, cones are separated from twigs and subsequently kilned to reduce the moisture content to about 10% (w/w) in a kiln, typically consisting of a perforated floor on which the hop cones are spread out and dried by forced circulation of heated air[32]. Drying is a critical point in the process and temperatures above 65°C may cause losses of hop acids and negatively affect the quantity and quality of hop oils. Upon kilning, cold conditioning of hops may be required to equally distribute the moisture content, which may vary amongst hop cones depending on their position in the bed during kilning[1,51]. Dried cones or whole hops are subsequently packaged in bales[1]. Quantities of hops are often measured in Zentners, which equals 50 kg[29,32].

1.3.2 Hop secondary metabolites

1.3.2.1 Chemical composition of hops

The term 'secondary metabolites' indicates substances that are formed in plants but do not participate in primary metabolic processes essential for life and development of the plant[1,52]. These secondary metabolites may be derived from components of any of the vital biochemical pathways or may simply be waste products modified to serve a useful purpose. Hops contain hundreds of secondary metabolites, comprising many different groups of organic compounds[1]. The major components that are found in dried hop cones are summarised in **Table 1-1.** Compounds occurring in the polyphenol fraction and in the hop oil specific for the hop plant have not been detected, whereas the hop acids have not been found in any other plant species[1]. From a brewer's perspective, of particular interest are the hop acids, particularly the α-acids, the hop essential oil components and the polyphenols. These three chemical classes of hop secondary metabolites are also regarded as 'the clue' to differentiate hop varieties[53,54]. In the next sections, more details will be given on the chemistry of hop acids and, in particular, the hop essential oil which is related to hoppy aroma of beer.

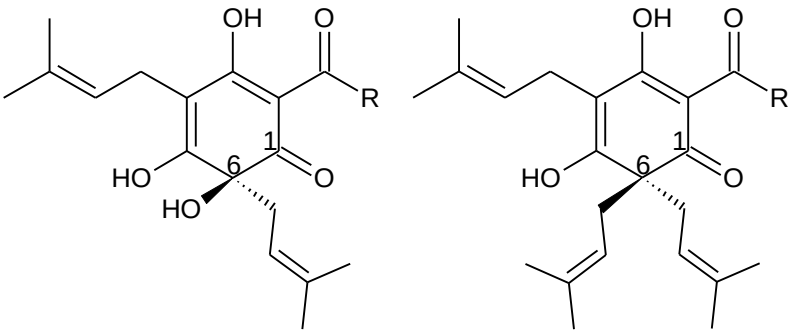
Table 1-1. Major components found in dried hop cones[1].

Major components	Concentration % (w/w)
α-Acids	2-19
β-Acids	2-10
Essential oil	0.5-3 (v/w)
Polyphenols	3-6
Monosaccharides	2
Amino acids	0.1
Proteins	15
Lipids and fatty acids	1-5
Pectins	2
Ash – salts	10
Cellulose – lignins	40-50
Water	8-12

1.3.2.2 Hop resins

Hop resins are a complex mixture of the so-called soft resins and hard resins, which are found in the lupulin glands. The total resin fraction is soluble in cold methanol and diethyl ether. Soft resins comprise the hexane-soluble fraction, whereas the hard resin fraction is insoluble in hexane[1]. Hard resins mainly consist of oxidation products of hop acids. The hop acids are part of the soft resin fraction and consist of two related series, the α-acids (humulones) and the β-acids (lupulones). These compounds are weak organic acids (pK_a of resp. 5.5 and 8), exhibit very poor solubility in water and have almost no bitter taste. The most important are the α-acids, since they are the precursors for the beer bittering principles (iso-α-acids)[1,46].

Hop α-acids (humulones) exist as 5 different analogues (isomers and homologues), *i.e.* humulone, cohumulone, adhumulone, prehumulone and posthumulone (see **Figure 1-4**).



Group R	α-acids	β-acids
$CH_2CH(CH_3)_2$	Humulone	Lupulone
$CH(CH_3)_2$	Cohumulone	Colupulone
$CH(CH_3)CH_2CH_3$	Adhumulone	Adlupulone
$(CH_2)_2CH(CH_3)_2$	Prehumulone	Prelupulone
CH_2CH_3	Posthumulone	Postlupulone

Figure 1-4. Analogues of α-acids and β-acids[1].

The major component of the α-acids mixture is humulone. Among different hop varieties, the relative amount of adhumulone is relative constant, whereas the relative amounts of humulone and cohumulone are variety-dependent (20-50%)[1,55]. Cohumulone has been associated with a poor hop quality (*i.e.* poor bitterness quality)[56], although this issue remains a matter of debate[1,57,58]. Furthermore, the cohumulone ratio has also been used as criterion for hop variety characterisation[59]. Pre- and posthumulone are only minor constituents[1].
The β-acids or lupulones also comprise five analogues which correspond to those of the α-acids (**Figure 1-4**). Due to the substitution of carbon atom C-6 with two 3-methyl-2-butenyl groups, the β-acids can, in contrast to the α-acids, not be isomerised to form bitter iso-α-acids (for the conversion of α-acids into iso-α-acids, see further). Moreover, β-acids are largely lost during the brewing process by precipitation. However, some oxidation products of the β-acids (*e.g.* hulupones) may survive the brewing process and contribute to beer bitterness[60].

The thermal isomerisation of the α-acids into the bitter iso-α-acids during wort boiling is probably the most important chemical conversion in brewing hop chemistry. This reaction, shown in **Figure 1-5**, has been studied into detail by De Keukeleire and Verzele[61] and Jaskula *et al.*[3]. The reaction occurs via an acyloin-type ring contraction as described by Steenackers *et al*.[62]. More particularly, the isomerisation of α-acids consists of deprotonation of the beta-tricarbonyl moiety, tautomerisation of the undissociated enol to the corresponding ketone and rearrangement of this ketone to an α-ketol system, leading to contraction of the ring. As a result, a chiral centre at the C4-atom is formed, giving rise to two diastereomeric isomerisation products: the *cis*-iso-α-acids (with the C5-atom in R-configuration) and the *trans*-iso-α-acids (with the C5-atom in S-configuration)[62]. As a result, 6 major iso-α-acids can be distinguished in beer (*i.e. trans*-isohumulone and *cis*-isohumulone, *trans*-isocohumulone and *cis*-isocohumulone, *trans*-isoadhumulone and *cis*-isoadhumulone)[61].
The *cis* to *trans* ratio depends on the reaction conditions. Under normal kettle boiling conditions the ratio is about 68:32 in favour of *cis*-compounds, which are energetically more likely to be formed[1,3,46,63]. A major problem in brewing practice is the poor solubility of the α-acids (40 mg/L at 25°C, 60 mg/L at 100°C) in the wort medium, thereby limiting their conversion. However, iso-α-acids are much more water soluble and they survive the boiling process to a great extent[1].

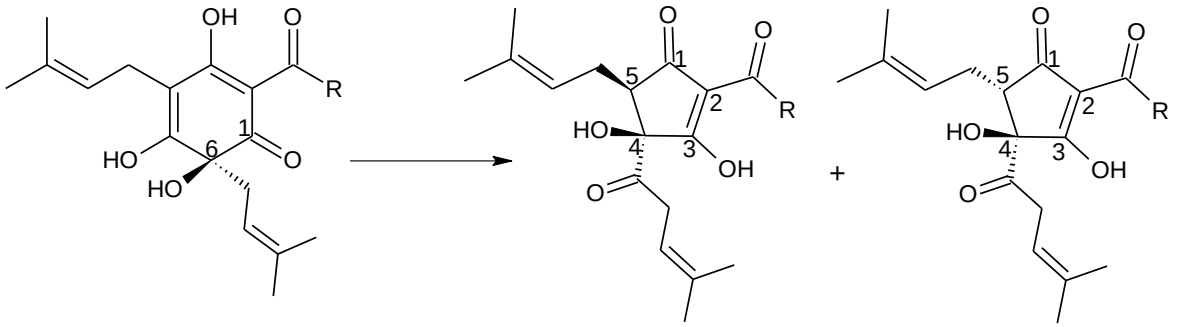


Figure 1-5. The isomerisation of hop α-acids into iso-α-acids[62].

Although iso-α-acids are the main bittering principles in beer, it has been reported that many other constituents (*e.g.* hop acid oxidation products) can contribute to beer bitterness[1,25,63]. However, iso-α-acids are by far the most important bitterness substances. Depending on the beer, their levels in beer vary between 10 and 80 mg/L, which is well above their taste threshold value in beer of around 5-7 mg/L[63,64]. The sensory properties of *cis*- and *trans*-iso-α-acids in both aqueous buffer solutions and unhopped beer have extensively been studied[64–70]. Apparently, in aqueous buffer solutions, the bitterness intensity of *cis*-isohumulones is almost twice as much as that of *trans*-isohumulones. On the other hand, *trans*-isocohumulone is slightly less bitter than *trans*-isohumulone, whereas the bitterness intensity of *cis*-isocohumulone is similar to *trans*-isohumulone. Summarised, *cis*-isomers are more bitter than their corresponding *trans*-isomers and isohumulones have a higher bitterness intensity compared to isocohumulones. Besides being the main bittering substances and their bacteriostatic activity, iso-α-acids also contribute to foam formation and stabilisation as well as cling properties and are involved in the formation of light struck flavour[70–74].

1.3.2.3 Hop essential oil

The hop oil represents a relatively small, volatile fraction of hops (0.5 – 3.0 v/w%). The complex mixture of volatiles is found, together with the hop acids, in the lupulin glands[1]. Seedless hops tend to contain more essential oil. The oils are produced in the hop late in ripening, after the majority of the resin has been laid down, which highlights the need for harvesting at the appropriate time[29]. Hop oil is an extremely complex mixture of volatiles since more than 400 compounds have been identified[75] and it has even been suggested, using advanced multidimensional chromatographic techniques, that over 1,000 different compounds may be present in hop oil[76]. In general, hop essential oil volatiles are classified into an apolar (hydrocarbon) fraction and a polar fraction, which includes both oxygenated compounds and organosulfur compounds[1].

Hydrocarbon fraction

The nonpolar hydrocarbon fraction makes up 40 to 80% of the total hop oil and, apart from a few simple alkanes, the compounds in this group are terpenoid in origin. These compounds, mostly mono- and sesquiterpene hydrocarbons (formula $C_{10}H_{16}$ and $C_{15}H_{24}$, respectively) can be acyclic, monocyclic, bicyclic and even tricyclic (in case of sesquiterpene hydrocarbons)[2]. The hydrocarbon fraction consist mainly of the monoterpene hydrocarbon β-myrcene **(1)** (**Figure 1-6**) and the sesquiterpene hydrocarbons β-caryophyllene **(2)**, α-humulene **(3)** and in some varieties β-farnesene **(4)**[1,2,43]. Besides the major monoterpene β-myrcene, monoterpene hydrocarbons include compounds such as β-pinene **(5)** and β-ocimene **(6)**[39]. According to Verzele, β-myrcene, α-humulene and β-caryophyllene can, together, even represent 80 to 90% of total hop essential oils[43]. The predominant monoterpene β-myrcene can make up 30% of the total oil. The (quantitatively) most important sesquiterpenes α-humulene and β-caryophyllene can account for up to 18-33% and 4-22%, respectively, of total hop oil[51,77]. Some of the sesquiterpene hydrocarbons characterise certain varieties of hops[78] (*e.g.* bergamotene (> 10 ppm) and β-farnesene (> 150 ppm) for cv. Saaz, Lublin and Styrie[79]), which is considered to be highly valuable for varietal discrimination[79,80], and, this is interpreted in terms of genetic control of the multitude of biosynthetic pathways.

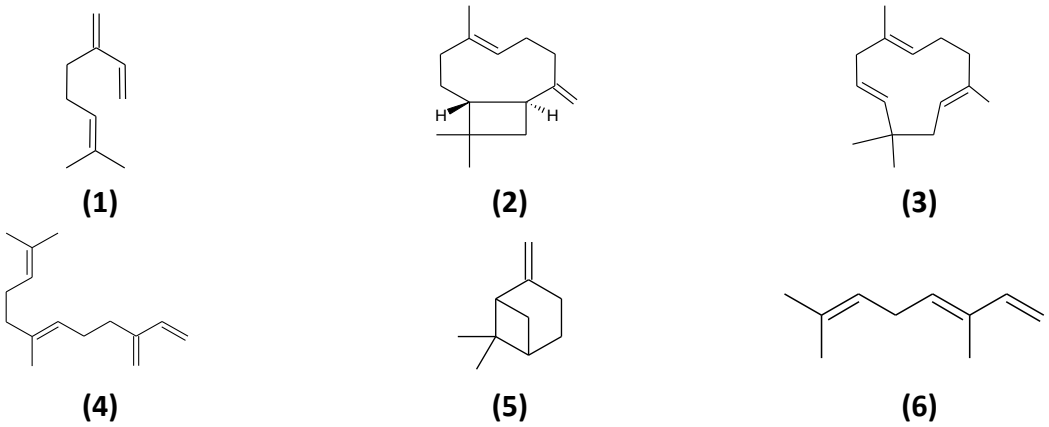


Figure 1-6. Examples of mono- and sesquiterpene hydrocarbons found in hop essential oil. (1) β-myrcene, (2) β-caryophyllene, (3) α-humulene, (4) β-farnesene, (5) β-pinene, (6) β-ocimene.

Terpene hydrocarbons are formed by isomerisation of activated isoprene units (isopentenyl diphosphate) (see **Figure 1-7**). Isopentenyl diphosphate-Δ-isomerase isomerises these isoprene units into 3,3-dimethylallyl diphosphate, whereupon the diphosphate group is removed to form a C5 carbocation that is further transferred to a second isopentenyl diphosphate molecule. As a result, geranyl diphosphate or neryl diphosphate, are formed and these molecules are precursors for monoterpenes (*e.g.* β-myrcene). Upon further transfer of another isopentenyl diphosphate molecule to geranyl diphosphate, a precursor for the sesquiterpenes, *i.e.* farnesyl diphosphate, is formed[52,81]. All hop varieties possess

cyclases which can transform (E,E)-farnesyl diphosphate into humulene and caryophyllene. An alternative pathway to form caryophyllene involves cyclisation of a suitable folded (Z,E)-farnesyl diphosphate, which is the probable precursor of the cadinenes, muurolenes, ylangenes and copaenes[77] (for examples, see **Figure 1-8**). These sesquiterpene hydrocarbons are also found in all hop varieties but their levels can significantly differ depending on the hop variety. Moreover, (E,E)-farnesyl diphosphate can be folded and cyclised to cyclodecadienyl cations which are precursors of germacrenes and eudesmanes (including selinenes)[77] (for examples, see **Figure 1-8**).

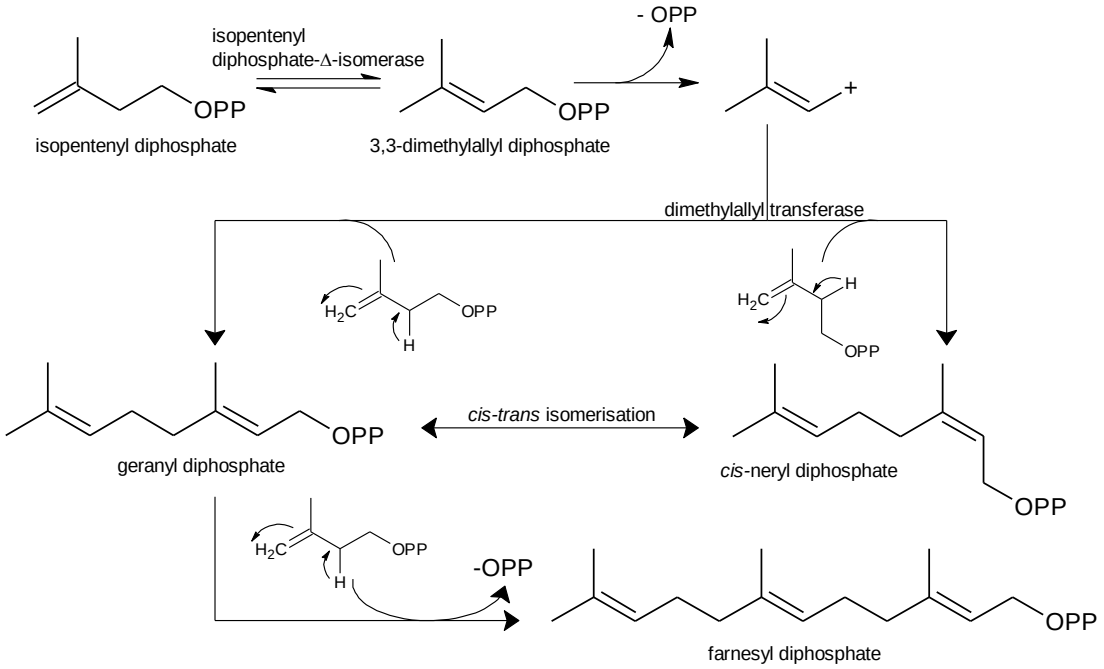


Figure 1-7. Biosynthesis of mono- and sesquiterpene precursors (adapted from Lermusieau and Collin[81]).

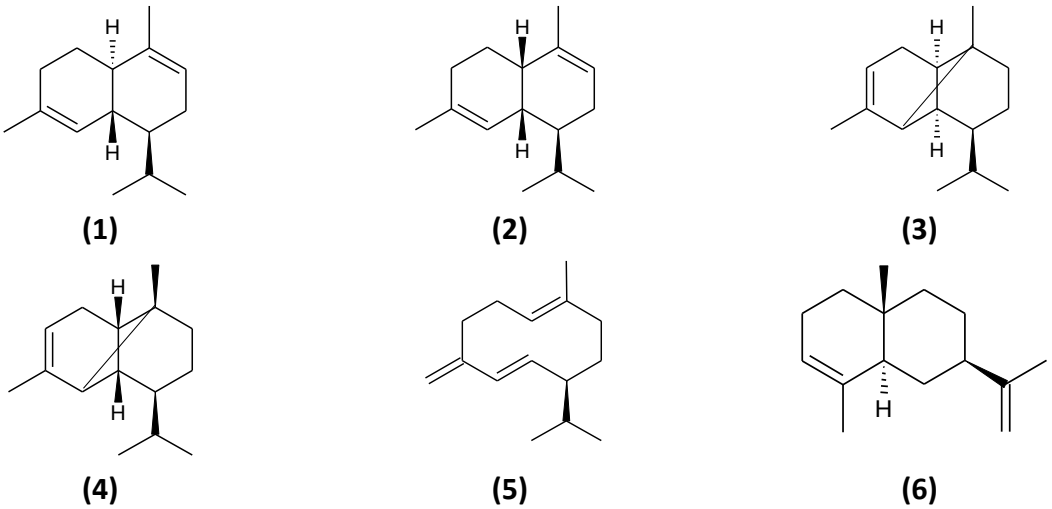


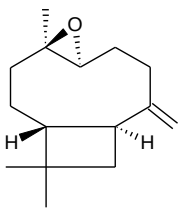
Figure 1-8. (1) α-cadinene, (2) α-muurolene, (3) α-ylangene, (4) α-copaene, (5) germacrene D, (6) α-selinene.

Oxygenated fraction

The oxygenated fraction represents 20 to 50% of the total hop oil and is an extremely complex mixture of epoxides, alcohols, ketones, aldehydes, acids, esters and 'miscellaneous oxygenated compounds'.

Epoxides. Epoxides are formed by autoxidation of sesquiterpene hydrocarbons. Epoxide levels in hop oil increase on hop storage[17,82] and since oxidation reactions take place rapidly, it is not uncommon to find humulene and caryophyllene epoxide in fresh hops[28]. Caryophyllene epoxide **(1)** (**Figure 1-9**) and humulene epoxide I **(2)**, II **(3)** and III **(4)** have been identified[17,51] and epoxides of aromadendrene **(5)**, alloaromadendrene **(6)** and β-selinene **(7)** have been identified in Hersbrucker Spät hops[78]. In addition, various humulene diepoxides, such as humulene diepoxide A **(8)**, have also been found in hops[10,83].

Alcohols. A series of straight and branched chain 1-alkanols has been reported to occur in hop oil. However, some of these alcohols may be artefacts from extraction or subsequent processing[51]. For example, 2-methyl-3-buten-2-ol, a degradation product of resin acids, accumulates in stored hops. However, the majority of alcohols found in hops are terpene alcohols. Linalool **(9)** is the major monoterpene alcohol identified in hops and can account for up to 1% of the oil. Isomeric compounds are geraniol **(10)** and α-terpineol **(11)**. A major distinction should be made between alcohols that are end-products of biosynthetic pathways (*e.g.* linalool, farnesol **(12)**, nerolidol **(13)**) and allylic terpene alcohols that are rearrangement products of terpene oxidation products (*i.e.* epoxides)[77]. Levels of the former group tend to decrease during hop storage whereas levels of the latter group increase. Examples of humulene and caryophyllene epoxide-derived allylic alcohols found in hops include humulenol II **(14)**[78,84], caryophylladienol (caryophylla-4(12),8(13)-diene-5-ol) **(15)** and caryophyllenol I (3Z-caryophylla-3,8(13)-diene-5α-ol) **(16)**[85]. However, non-allylic sesquiterpene alcohols such as humulol **(17)**[82] and caryolan-1-ol **(18)**[78] have also been detected in hops. Moreover, several non-humulene/caryophyllene-derived bicyclic alcohols have been found in hops, such as γ-eudesmol **(19)**, epicubenol **(20)**, δ-cadinol **(21)**, τ-cadinol **(22)** and α-cadinol **(23)**[82]. Up to date, it remains unclear whether these compounds are derived from selinene and cadinene epoxides[82] or are instead biosynthesised by the hop plant[18].



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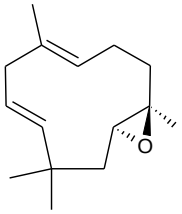

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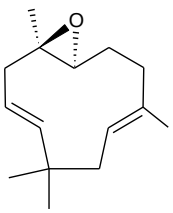

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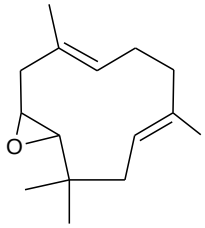

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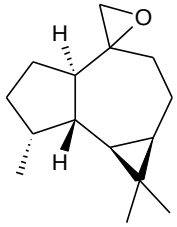

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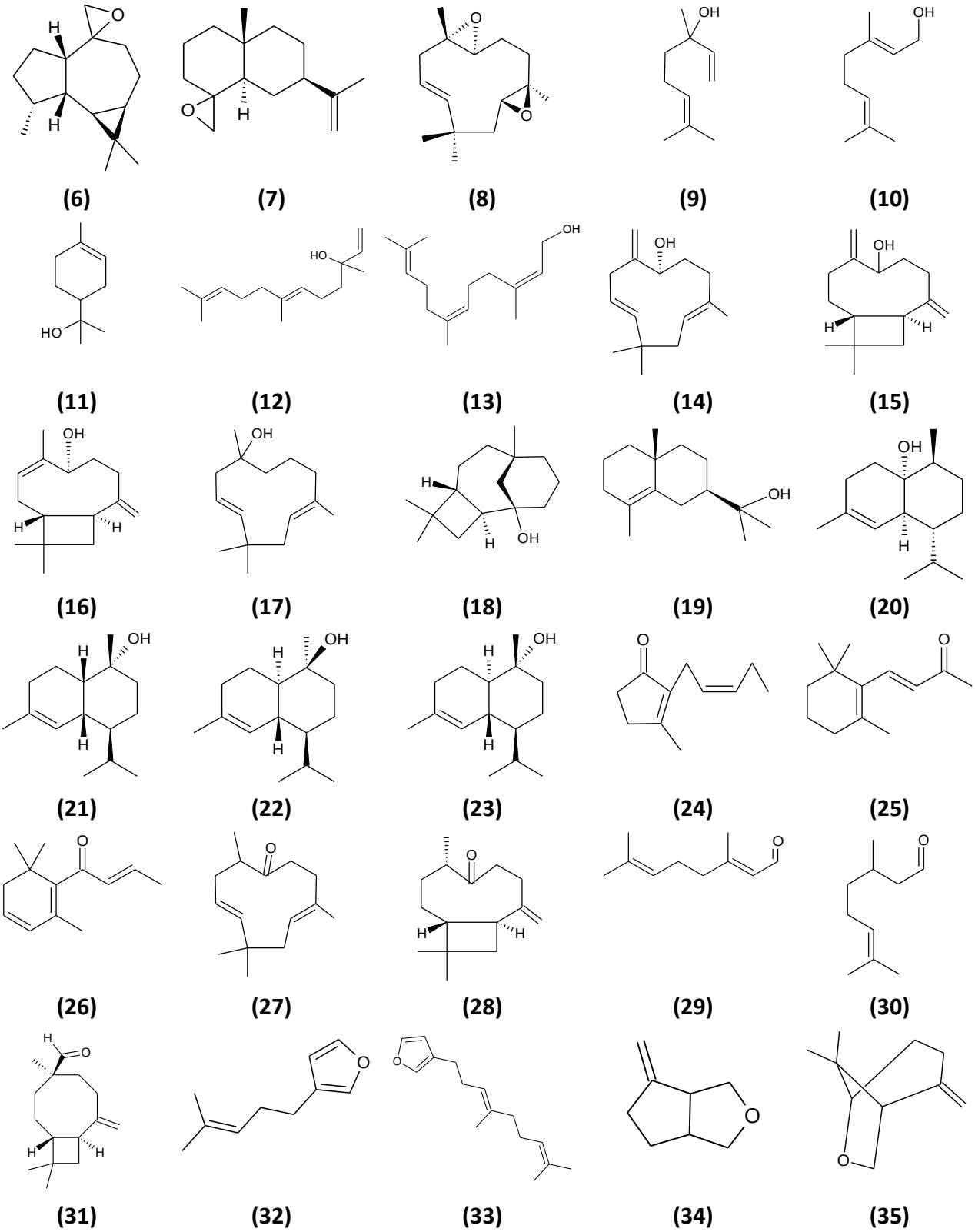


Figure 1-9. Examples of oxygenated compounds found in hop essential oil. Caryophyllene oxide (1), humulene epoxide I (2), II (3) and III (4), aromadendrene epoxide (5), alloaromadendrene epoxide (6), β-selinene epoxide (7), humulene diepoxide A (8), linalool (9), geraniol (10), α-terpineol (11), farnesol (12), nerolidol (13), humulenol II (14), caryophylla-4(12),8(13)-diene-5-ol (15), 3Z-caryophylla-3,8(13)-diene-5α-ol (16), humulol (17), caryolan-1-ol (18), γ-eudesmol (19), epicubenol (20), δ-cadinol (21), τ-cadinol (22), α-cadinol (23), *cis*-jasmone (24), β-ionone (25), β-damascenone (26), humuladienone (27), 4S-dihydrocaryophyllene-5-one (28), citral (29), citronellal (30), 6(5→4)-abeo-caryophyll-8(13)-en-5-al (31), perillene (32), dendrolasin (33), hop ether (34), karahana ether (35).

Ketones. Ketones are primarily known as derivatives from the β-oxidation of fatty acids[86]. A large number of methyl ketones have been reported to occur in hop oil[51]. A homologous series from 2-heptanone to 2-heptadecanone are found in almost all varieties and also branched and unsaturated ketones have been found, although the precise position of branching or unsaturation is in many cases not specified[51,75,77,87]. Moreover, Moir[77] identified alkane-2,4-diones in Wye Target hop oil. Also non-aliphatic ketones such as *cis*-jasmone **(24)**[88], and, the nor-carotenoids β-ionone **(25)** and β-damascenone **(26)** have been reported[82]. Humulene and caryophyllene-derived ketones reported in hops include humuladienone **(27)**[89] and 4S-dihydrocaryophyllene-5-one **(28)**[85], the latter being a rearrangement product of caryophyllene epoxide.

Aldehydes. Besides a few saturated and unsaturated aliphatic aldehydes, the monoterpenoid aldehydes citral **(29)** and citronellal **(30)** have also been reported as hop oil constituents[77]. Until now, 4,10,10-trimethyl-7-methylenebicyclo[6.2.0]decane-4-carbaldehyde (or 6(5→4)-abeo-caryophyll-8(13)-en-5-al) **(31)**, formed by rearrangement of caryophyllene oxide, is the only C_{15}-derived aldehyde compound identified so far[85].

Acids. Acids are only present in trace amounts in the essential oil of fresh hops and have, in most cases, not been strictly classified as essential hop oil constituents since they are supposed to originate from oxidation of the resin acids (*e.g.* 2-methylbutyric acid)[51,77]. Decanoic acid is often accompanied by 4-decenoic acid[77] and also several branched chain acids have been isolated from hops[90].

Esters. More than 70 esters have been identified as hop oil components. A homologous series of methyl esters from hexanoate to dodecanoate is found, as well as a number of branched chain and unsaturated methyl esters. However, in many cases, the point of position of branching or unsaturation is, comparable to the methyl ketones, not known[51]. Esters of terpene alcohols, such as geraniol, nerol and linalool have also been identified (*e.g.* geranyl acetate)[77].

Miscellaneous. The last group of oxygenated hop oil compounds consists of various compounds that can not be classified in the chemical classes discussed above. Such compounds are indicated with the term 'miscellaneous'. Examples are furan derivatives (*e.g.* perillene **(32)** and dendrolasin **(33)** [91]) and cyclic ethers (*e.g.* hop ether **(34)** and karahana ether **(35)**).

Organosulfur compounds

Finally, hop oils often contain a range of organosulfur compounds. These include thiols, sulfides, polysulfides, thioesters, thiofenes and episulfides[1]. Concentrations of sulfur compounds in oils rarely exceed 0.1%[92]. Although sulfur compounds are present in very low quantities in hops, some have flavour thresholds of only a few parts per billion or even lower[1]. The origin of many sulfur compounds is unclear and some of them are probably artefacts formed by steam distillation of the hops prior to GC-analysis.

- Methyl thioesters are prominent in steam distilled hop oils[86,93,94]. They are not artefacts of the boil used in the extraction procedure since they are present in cold extracts of hops. Levels of thioesters in hops appear to be determined by variety and local growing conditions, as well as the kilning treatment for drying the cones[95]. Thioesters are found in green hops and possibly originate from thiolysis of acyl coenzyme A by methanethiol (a methionine degradation product)[86,95].
- The occurrence of 3-alkylthiophenes in the oil distilled from occasional batches of hops can be traced to abnormally high residual sulfur levels[96]. 3-Methylthiophene (see **Figure 1-10**) **(1)** and 3-(4-methylpent-3-enyl)-thiophene **(2)** have both been detected in hops[95].
- The presence of dimethyl trisulfide (DMTS) **(3)** in certain steam distilled hop oils was shown to originate from a labile precursor, almost certainly S-methylcysteine sulfoxide **(4)**, during steam distillation of hops[93]. Besides dimethyl trisulfide, many other methyl sulfides have been identified in hops, amongst them dimethyl sulfide (DMS) **(5)**, 3,3-dimethylallyl methyl sulphide **(6)**, methylthiohumulene **(7)** and dimethyl disulfide (DMDS)[95] **(8)**.
- Episulfides such as 1,2-epithiohumulene **(9)**, 4,5-epithiohumulene **(10)** and 4,5-epithiocaryophyllene **(11)** have been identified in hops[95]. Episulfides are formed when sulfur and the appropriate sesquiterpene are stored together with exposure to light or heated together in boiling water. Myrcene is another of the major hop oil constituents which reacts with sulfur under mild conditions[97].

Although sulfur compounds are characterised by sensory impressions with a negative connotation (*i.e.* 'cheesy', 'cooked vegetable', 'sulfury', 'soapy', 'rubbery', 'onion', 'burnt')[95], researchers detected sulfur compounds with a positive sensory impact. Kishimoto and coworkers identified 4-mercapto-4-methylpentan-2-one (4MMP) **(12)** as main contributor to the fruity, black-currant aroma in USA, Australian and New Zealand hop cultivars. The 4MMP content was highest in cv. Simcoe, followed by cv. Summit, Apollo, Topaz, Cascade pellets, and also differed among crop years[98]. Two thiols, 3-sulfanyl-4-methylpentan-1-ol (3S4MP) **(13)** and 3-sulfanyl-4-methylpentyl acetate (3S4MPA) **(14)** have been identified in cv. Nelson

Sauvin, which impart the characteristic exotic fruit-like, white wine-like flavour-note in beers brewed with this hop variety[99].

Recently, Gros *et al*.[100] assessed wheter S-cysteine conjugates migh constitue part of the hop thiol potential. These authors found, for the first time, evidence for the presence of the S-cysteine conjugate 3-S-(1-hydroxyhexyl)cysteine **(15)** in a Cascade hop extract[100]. In their subsequent study[101], these authors enzymatically released odourant polyfunctional thiols from cysteine conjugates in hop hydroalocholic extracts. The Cascade hop extract exhibited the highest bound 3-sulphanylhexan-1-ol (grapefruit-like) **(16)** potential, while both Tomahawk and Nelson Sauvin cultivars were confirmed to be important sources of bound 3-methyl-2-butene-1-thiol (skunky-like) **(17)**, 3-sulphanylpentan-1-ol **(18)** and 4-sulphanyl-4-methylpentan-2-one (box-tree-like) **(19)**. In addition, also CO_2 extracts proved to contain cysteine conjugates[101]. These S-cysteine conjugates can be chemically degraded in beer to release their corresponding thiols. The conversion rates are however relatively low[102].

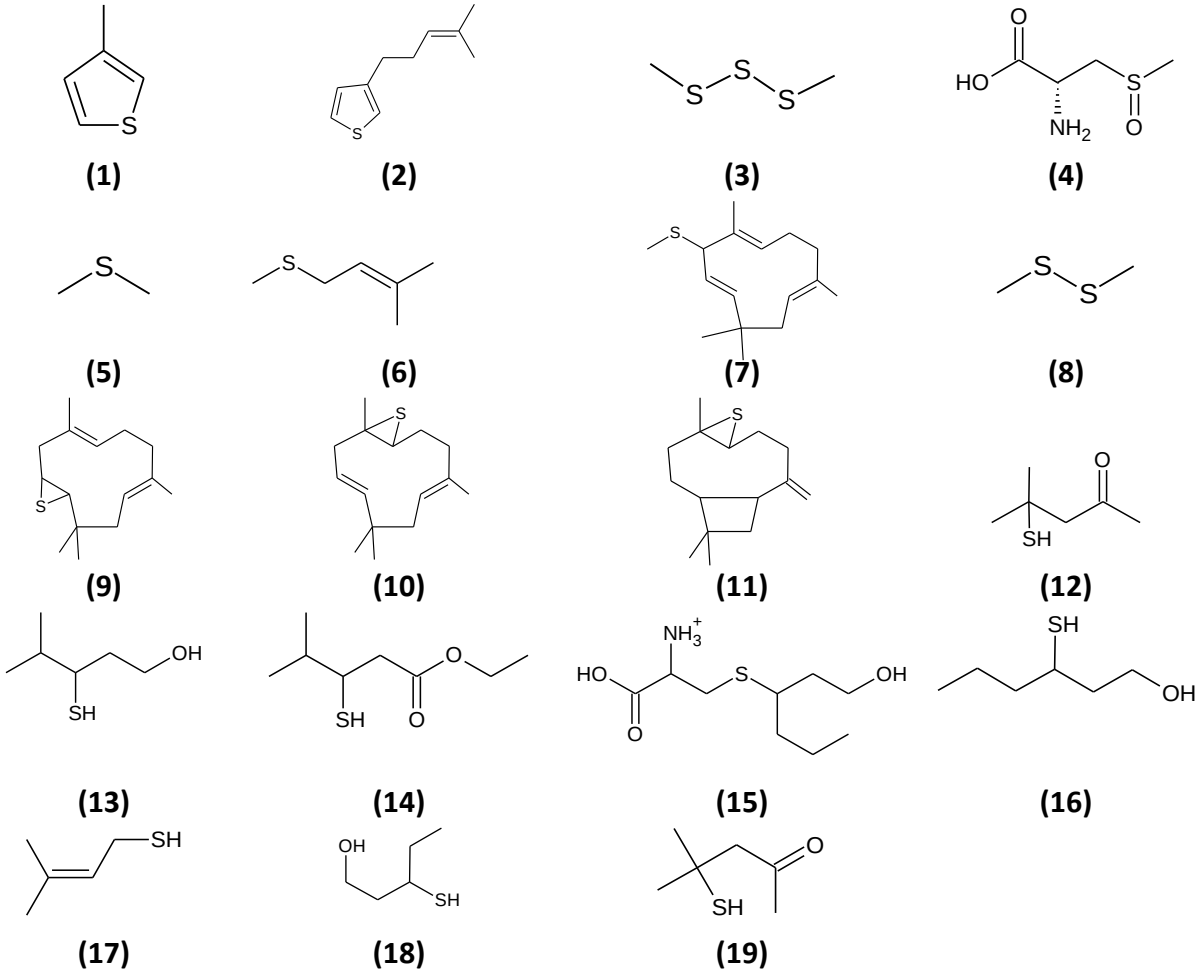


Figure 1-10. Examples of sulfur compounds found in hop essential oil or as S-cysteine bound conjugates. (1) 3-methylthiophene, (2) 3-(4-methylpent-3-enyl)-thiophene, (3) DMTS, (4) S-methylcysteine sulfoxide, (5) DMS, (6) 3,3-dimethylallyl methyl sulphide, (7) methylthiohumulene, (8) DMDS, (9) 1,2-epithiohumulene, (10) 4,5-epithiohumulene, (11) 4,5-epithiocaryophyllene, (12) 4MMP, (13) 3S4MP, (14) 3S4MPA, (15) 3-S-(1-hydroxyhexyl)cysteine, (16) 3-sulphanylhexan-1-ol, (17) 3-methyl-2-butene-1-thiol, (18) 3-sulphanylpentan-1-ol, (19) 4-sulphanyl-4-methylpentan-2-one.

1.3.3 Hop varieties

Traditionally, hop varieties have been classified into 'aroma' and 'bitter' types, depending on their α-acid content and flavour characteristics (see **Table 1-2**). Hops with high α-acid contents above 6 w/w%, a moderate to high cohumulone content and little regard to the aroma quality are considered bitter hops, whereas aroma hops are typically characterised by α-acid contents below 5 w/w%, a characteristic ratio of the major essential oil compounds (*i.e.* α-humulene, β-caryophyllene and β-farnesene), a low cohumulone content and a distinctive aroma quality[1,103]. As bitter hops with higher α-acid content were selected for breeding, a significant increase in the concentration of α-acids occurred in the newer varieties. Therefore, high alpha hops were introduced as a new classification. These hop varieties are generally considered as bitter varieties containing more than 13 w/w% α-acids[103]. The classification between bitter and aroma hops is however somewhat misleading because every hop variety contains hop oil and hop acids and some hop varieties are characterised by an excellent aroma and a relatively high α-acid content[1]. Such hop varieties, combining an intermediate resin level together with an appealing aroma, are nowadays described as 'dual-purpose' hops. Aroma varieties (*e.g.* cv. Saaz) command higher cost prices due to their desired hop oil aroma. They are, however, seldom used as the sole source of bitterness and aroma in beer. Usually, cheaper higher-α-acid hop is used at the onset of wort boiling to provide the bulk of the bitterness, whereas the prized aroma varieties are added late to the boil[29].

Very recently, a new type of hop is emerging as a result of the flourishing USA craft brewing industry and its appetite for high hop impact in their beers. As a result, hop varieties with bold, atypical aromas, often exhibiting 'non-classic' hop aromas such as high citrus, fruity and even tropical fruit, have been commercially developed. Since these hop varieties significantly differ from the classic aroma hops and their α-acid content widely varies, The Barth-Haas Group (the world's largest supplier of hops and hop products) has classified these hop varieties as flavour hops[103].

Some classic European hop varieties such as Hallertauer Mittelfrüh, Tettnanger, Hersbrucker etc., are noted as noble aroma hops because of their ability to impart 'kettle hop' or 'noble hop' flavour to the final beer[10,104]. Usually, such aroma hop varieties comprise relatively high humulene levels[28,105,106]. These traditional aroma hop varieties may be slightly aged by the brewer to increase the level in sesquiterpene oxidation products or 'noble' hop aroma compounds to enhance their contribution to the final beer flavour[104].

Table 1-2. Classification of hop varieties[1,103]. Varieties in bold impart spicy/herbal notes to beer, related to classic European noble aroma.

Country	Aroma hops	Bitter hops	High alpha hops	Dual-purpose hops
Australia	Ella, **Helga**, Summer, **Sylva**		Pride of Ringwood, Super Pride	Galaxy, Topaz
Austria	**Styrian Gold**			
China			Marco Polo	Tsingdao Flower
Czech Republic	**Bohemie**, Kazbek, Premiant, **Saaz**, Saaz Late, **Sladek**	Rubin		Agnus
France	Aramis, Strisselspalt, Triskel			
Germany	Hallertau Blanc, **Hallertau Mittelfrüh**, **Hersbrucker**, Hüll Melon, Mandarina Bavaria, Opal, Perle, Polaris, Saphir, Smaragd, **Spalt Spalter**, Spalter Select, **Tettnanger**, **Tradition**		Herkules, Magnum, Merkur, **Taurus**	Northern Brewer
Japan				Sorachi Ace
New Zealand	**Motueka**, Nelson Sauvin, Pacifica, Riwaka		Pacific Gem, Pacific Sunrise	New Zealand Hallertau, Pacific Jade
Poland	Limbus, Lomik, **Lublin**, Sybilla	Junga, Marynka, Oktawia, Pulawski, Zbyszko, Zula	Magnat	
Slovenia	Aurora, Bobek, Celeia, Harmonie, **Styrian Savinjski Golding**			Bor, Extra Styrian Dana
South Africa		Southern Brewer		Southern Dawn, Southern Promise, Southern Star
UK	Bramling Cross, East Kent Golding, Endeavour, First Gold, Fuggle, Sovereign, Whitbread Golding	Pilgrim, Pilot, Target	Admiral	Boadicea, Brewers Gold, Northdown, Pioneer, Progress, Wye Challenger
USA	Ahtanum, Amarillo, Calypso, Cascade, Centennial, Citra, Crystal, Delta, Liberty, Mosaic, Mount Hood, Palisade, **Santiam**, Sterling, **Ultra**, **Vanguard**, Wilamette	Cluster	Apollo, Bravo, Chelan, Columbus, Comet, Galena, Millennium, Newport, Nugget, Summit, Super Galena, Tomahawk, Warrior, Zeus	Chinook, Glacier, Horizon, Simcoe

1.3.4 Hop products

In traditional brewing practice, hops were used in their unprocessed form. After picking and drying, whole hop cones are compressed into rectangular bales for storage and transport. However, some problems are associated with the use of whole hops such as difficulties for automatic dosage due to low bulk density and stickiness, degradation during storage, heterogeneous α-acid content, low concentration of brewing components, the presence of chemical residues and a low utilisation of α-acids (*i.e*. the percentage of α-acids that is isomerised and remains in the finished beer) in brewing, typically in the range of 30-40%[1].

To partially solve these problems and meet particular needs of the brewer, a large series of hop products has been developed and is nowadays used in brewing practice. These products offer many advantages such as an improved preservation of the brewing value, the ease of handling, higher utilisation of the brewing principles and enhanced consistency of bitterness and hoppy aroma in the final beer[1,107]. Today, more than 20 different types of hop products are available, which can be classified into two major categories. Conventional or non-isomerised hop products comprise non-isomerised pellets and hop extracts[1,108,109], whereas advanced hop products are generally more refined preparations to provide bitterness or hoppy aroma.

1.3.5 Economic aspects

According to The Barth Report 2014-2015[110], the worldwide beer production in 2014 was 1.96 billion hL (market revenue of 494.4 billion $), of which ca. 523 million hL was brewed in Europe and ca. 18 million hL in Belgium. In Asia, 704 million hL was brewed, followed by (North and South) America where 572 million hL of beer was produced. Although hops are only a minor ingredient for beer production, 96,477 tonnes of hops were produced in 2014. When expressed in α-acid weight, 9,227 tonnes of hop α -acids were produced. The area under hop cultivation for this production is 47,766 ha. The crop share of fine aroma hops (*i.e.* noble European varieties), aroma hops and bitter hops (inclusive high alpha hops) was determined at 11.6%, 31.5% and 56.9%, respectively. By far, the most important hop growing countries are the U.S. and Germany, followed by China and the Czech Republic, which produced 31,454; 27,554; 7,194 and 5,330 tonnes of hops, respectively[110]. According to Biendl[24], in 2009, 97% of hops was used in the brewing industry. Of all the hop products used in the brewing industry, a share of 2%, 20%, 60% and 18% is assigned to hop cones, non-isomerised extracts, pellets and isomerised products, respectively. In other words, although the use of isomerised products is increasing (18% in 2009 compared to 5% in 1994), ca. 80% of the applied hop products are still conventional[24]. These data underline the economic importance of the hop industry and, in particular, of conventional hop products.

1.4 Hop aroma

All plant material contains volatile compounds that often have a typical smell[43]. The characteristic odour/aroma of hops is imparted by the volatile hop essential oil fraction. The hop essential oil composition of hops mainly depends on the hop variety, although agronomic factors such as growth place and seasonal aspects may also influence the hop oil composition[111–113]. Thus, aroma properties may differ among hop varieties, which can be attributed to variations in the hop essential oil composition[114,115]. It is well accepted that only a limited number of volatile compounds are responsible for the overall aroma of foods or their raw materials[116–118]. Such compounds can be differentiated from the bulk of the rather odourless compounds by applying GC-olfactometry and odour dilution techniques (CHARM analysis (combined hedonic aroma response method) and AEDA (aroma extract dilution analysis)) or the odour activity value (OAV) concept[118]. In AEDA, samples are evaluated by the panellists in increasing dilution order and the impact of an odour-active compound is given by its dilution factor (FD) value. On the other hand, in CHARM analysis the dilutions are presented to the panellists in a randomised order, avoiding bias introduced by the knowledge of the dilution being analysed[119]. The odour activity value (OAV) is calculated as the ratio between the concentration in a sample and the threshold concentration of this substance and gives a measure of importance of a specific compound to the odour of a sample. Guadagni *et al.*[9] applied the latter technique and found that the major hydrocarbon fraction of hop essential oil accounted for 69% of the total odour activity, whereas the minor oxygenated fraction accounted for 34%. The authors found that β-myrcene, β-caryophyllene and α-humulene represented 58%, 1.6% and 1.5% of the total hop aroma, respectively. The contribution of linalool was only 0.3%[9].

β-Myrcene imparts the pungent smell to fresh hops. After a few months it has disappeared through air oxidation and by evaporation[43]. Generally, it is felt that β-myrcene is an undesirable feature in hop oil[29]. In general, high quality aroma hops are characterised by a high content in β-caryophyllene (C) and α-humulene (H) and low levels of β-myrcene[1,50]. Prized European aroma hops, which are known for their potential to impart 'kettle hop' aroma when used in brewing, tend to contain relatively high levels of α-humulene[27,28,105,106]. Kralj *et al.* found a negative correlation between β-myrcene and European hop aroma[106]. On the other hand, α-humulene, β-caryophyllene, carvone (see **Figure 1-11**, **(1)**), methyl 4-decenoate, 2-undacanone, β-farnesene, and humulene epoxide I are correlated with European hop aroma[106], and, furthermore, European hop varieties are characterised by a high H/C ratio[28,106]. Moreover, the quality of the Saaz variety, a typical European noble aroma hop, might be related to a limited amount of sulfur compounds, compared to *e.g.* the Challenger variety which is characterised by large amounts of sulfur compounds[120].

In general, the terpenic fraction found in raw hop oil typically produces citrus, herbal, spicy or woody aromas. Terpene alcohols (linalool, geraniol, farnesol) typically produce a floral aroma. The oxygenated fraction is further made up of esters and ketones which tend to produce fruity, floral, and waxy aromas[121]. Aged hops can sometimes be responsible for cheesy flavours; these are due to acids, such as 3-methylbutanoic acid, which originates from acyl side-chains of resin constituents such as the α-acids[82].

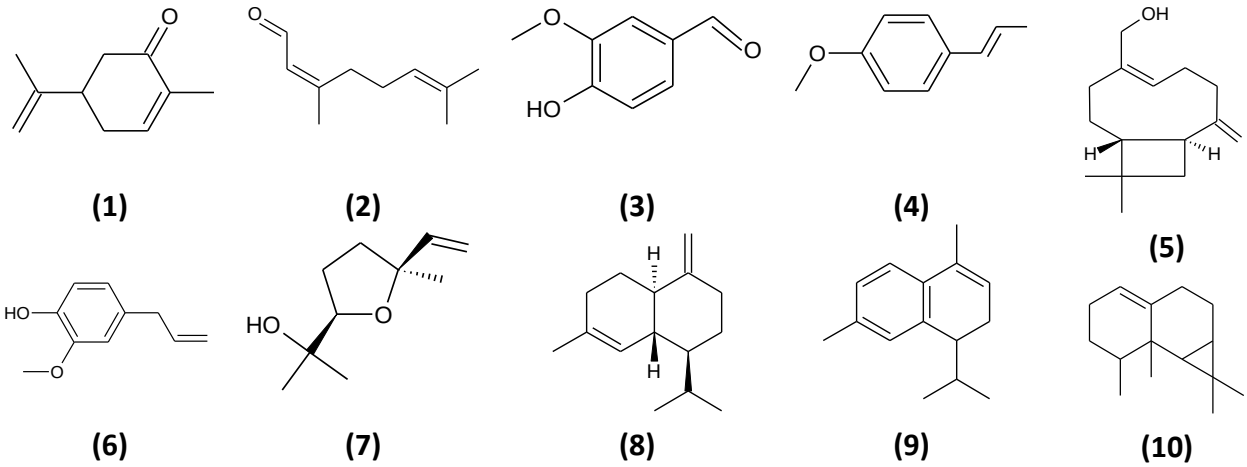
Over the last decades, much research has been carried out to relate particular sensory properties of hop cones and pellets to individual volatiles using gas chromatography-olfactometry (GC-O). Sanchez *et al.*[26] evaluated the oxygenated fractions of Hallertauer Mittelfrüh and two USA hop varieties using GC-O. The authors detected nine odour-active substances in all three varieties, three of which could be identified as linalool, neral **(2)** and humulene epoxide III, and, proposed linalool and oxidation products of β-caryophyllene and α-humulene to contribute significantly to the overall odour of all three hop varieties[26].
Steinhaus and Schieberle[122] found 23 potent aroma compounds in the hop variety Spalter Select. The most potent aroma constituents were *trans*-4,5-epoxy-E-2-decenal ('metallic note'), linalool ('flowery') and β-myrcene ('geranium-like'). (Z)-3-hexenal was characterised as a further key odourant rendering an additional green aroma to fresh hops[122].
Steinhaus *et al.*[4] compared the most odour-active volatiles in different hop varieties (cv. Hallertau Perle, Hallertau Hersbrucker Spät, Slowenian Golding, Hallertau Smaragd and US Cascade) using AEDA GC-O. The authors detected 38 odour-active zones in the different varieties. For all varieties investigated, β-myrcene, followed by linalool, showed the highest flavour-activity and were characterised by 'geranium' and 'citrus/bergamot' odours, respectively. Furthermore, 3-methylbutanoic acid (described as 'cheesy'), 2-isopropyl-3-methoxypyrazine, vanillin **(3)**, anethole **(4)** and geraniol proved significant flavour-activity in all five varieties. 4-Methyl-4-sulfanylpentan-2-one (4MMP) was identified as odour-active compound responsible for the black-current-like odour note of US Cascade hops[4].
Van Opstaele *et al.*[123] recorded 13 odour-active regions upon GC-O analysis of a floral hop essence cv. Spalter Select. β-myrcene ('fresh hops') and 2-undecanone ('floral/citrus') were detected by all assessors, suggesting that these compounds are high character impact compounds of the floral essence. In addition, major contributors to the odour of this essence are *cis*-β-ocimene ('green, floral'), nonanal ('citrus') and perillene ('citrus/lemon'). Other character impact compounds of the floral hop essence cv. Spalter Select are methyl octanoate ('fruity'), methyl 4-methyloctanoate ('citrus'), ethyl nonanoate ('fruity'), 2-dodecanone ('citrus') and methyl 3-nonenoate ('floral, citrus, green'). In particular, the importance of β-myrcene and 2-undecanone for the fresh hop and 'floral/citrus' characters

of this essence was proven, and, *cis*-β-ocimene, 2-undecanone, 2-dodecanone and several esters were reported for the first time as impact odourants of hop aroma[123].

Eyres *et al.*[114] characterised odourants in the spicy fraction of hop essential oil cv. Target, Saaz, Hallertauer Hersbrucker and Cascade. Odour-active compounds were tentatively identified using comprehensive two-dimensional gas chromatography (GCxGC) combined with time-of-flight mass spectrometry (TOFMS). An intense 'woody' odour was determined to be the most potent odourant in three of the four spicy fraction samples. The volatile responsible was identified as 14-hydroxy-β-caryophyllene **(5)**. Other important odourants identified were geraniol, linalool, β-ionone and eugenol **(6)**. Also β-damascenone, Z-linalool oxide **(7)**, pentyl 3-methylbutanoate, ethyl 3-methylbutanoate and 3-methylbutanoic acid ('goaty, sweaty, cheesy') were detected among odour-active compounds[114].

Recently, Van Opstaele *et al.*[124] identified 2-undecanone ('citrus'), 2-tridecanone ('green, woody'), γ-cadinene **(8)** co-eluting with α-calacorene **(9)** and calarene **(10)** ('spicy, woody'), humuladienone/caryolan-1-ol ('green'), a caryophyllene oxide enantiomer ('green, spicy') and humulene epoxide II, co-eluting with 2 unidentified compounds ('green, hay-like') in odour-active intervals detected upon GC-olfactometry of a spicy hop essence cv. Spalter Select. They concluded that because of the very complex chemical composition of the essence, it is not possible to identify those compounds causing the perceived odours, due to chromatographic coelution and the lack of authentic reference compounds[124].

Hop oils can also be a source of flavour-potent organosulfur volatiles, which give rise to unpleasant sulfury flavours[125]. Nevertheless, sulfur compounds can also have a positive and characteristic impact on aroma properties of particular hop varieties. The thiols 3-sulfanyl-4-methylpentan-1-ol (3S4MP) and 3-sulfanyl-4-methylpentyl acetate (3S4MPA) were found to be at the origin of the 'white wine-like' aroma of cv. Nelson Sauvin[99,126,127]. Kishimoto *et al.* discovered that 4-mercapto-4-methylpentan-2-one (4MMP), a potent odourant in American, Australian and New Zealand cultivars, contributes to the fruity aroma of these hops[12].



(1) **(2)** **(3)** **(4)** **(5)** **(6)** **(7)** **(8)** **(9)** **(10)**

Figure 1-11. Structures of hop aroma compounds. Carvone (1), neral (2), vanillin (3), anethole (4), 14-hydroxy-β-caryophyllene (5), eugenol (6), Z-linalool oxide (7), γ-cadinene (8), α-calacorene (9), calarene (10).

1.5 Changes in the hop essential oil composition

1.5.1 Modifications prior to brewing

1.5.1.1 Drying

After harvest, hop cones are usually dried by hot air. Howard and Slater[128] found that drying of hops generally reduced the total amount of essential oil. Narziss and Forster[129] reported losses of hop essential oil of up to 50% during kilning when higher temperatures were applied. Most of the volatiles investigated, for example, monoterpenes, sesquiterpenes, linalool, and esters, were decreased. However, an increase in oxidation products of sesquiterpenes and of compounds proposed as degradation products of hop bitter substances was also observed[129]. Sharpe and Laws[51] observed a reduction of the monoterpenoid level up to 40-50%, and a reduction of 20-40% for the sesquiterpenes was found, depending on the temperature used for drying, which was ascribed to both evaporation losses and oxidative processes. Therefore, in view of conserving hop aroma quality, a high air flow through the drying kiln has been recommended to shorten the contact time with hops[51]. Steinhaus and Schieberle[122] concluded that (Z)-3-hexenal, responsible for the 'green' and 'grassy' smell of fresh hops, is significantly degraded during drying of the hop cones[122]. Kilning also impacts levels of sulfur compounds, since a significant increase in the thioester level in hop essential oil up to 40% was observed by Seaton and Moir as a result of the drying process[86]. Drying temperatures which do not exceed 65°C are recommended in order to avoid damage of the hop cones and, next to losses of α-acids, changes in the quality of hop oils[1].

1.5.1.2 Processing

After drying, hops can be processed into different hop products, *e.g.* pellets or extracts, which might also affect the hop essential oil composition. For example, ethanolic extracts show lower terpene hydrocarbon levels compared to unprocessed hops. However, the terpene hydrocarbon spectrum of hop oil volatiles in supercritical carbon dioxide extract is nearly identical to unprocessed hops. When comparing ethanolic and carbon dioxide extracts, esters and ketone levels appear higher in the latter, whereas oxygenated sesquiterpenoid amounts are comparable. Extracts show a good storage stability compared to natural hops since sesquiterpene oxidation product levels do not increase upon storage[130].

1.5.1.3 Storage and ageing

Natural hops are in theory less prone to volatilisation and oxidation of hop essential oil volatiles since the lupulin glands remain intact, whereas processes such as pelletizing disrupt

the membrane surrounding the lupulin gland's content. However, hop products such as pellets are vacuum packaged and their higher density facilitates cold storage. As a result, hop products usually have a prolonged aroma quality stability. Nevertheless, even upon storage of hop cones at 0°C, the content of the major terpenes (β-myrcene, β-caryophyllene, α-humulene, β-farnesene) shows a relatively fast decrease[79,112]. Sharpe and Laws[51] reported a decrease of up to 20% in the level of hop oil monoterpenoids upon storage of frozen undried hops[51].

Tressl *et al.* made a major contribution to this issue by investigating changes in hop oil composition upon storage of hop cones[82]. Spalter hops were stored at 0°C and the hop essential oil volatile composition was investigated via GC-MS after 2 months and after a storage period of 3 years. The hop oil content showed a remarkable decrease upon storage. The amount of all terpenes decreased considerably, and this decrease was ascribed to decomposition by oxidative reactions and by polymerisation. Amongst the oxygenated compounds, the level of esters remained nearly constant and only the loss of unsaturated components was considered significant. The amount of unsaturated ketones decreased, whereas a series of humulone- and lupulone-derived ketones and ketones which may be formed by oxidative fatty acid degradation increased considerably upon storage. Aldehydes, derived from oxidative degradation of linoleic, linolenic and oleic acids were not found in fresh hops but only in stored hops. Most of the oxygenated sesquiterpenoids showed a pronounced increase (*e.g.* caryophyllene epoxide and humulene epoxide I and II). Among terpene alcohols, linalool, α-terpineol, caryolan-1-ol, humulol and humulenol II were strongly concentrated. The authors also identified for the first time in stored hops β-ionone and β-damascenone, known as oxidative degradation products of β-carotene[82].

Lam *et al.*[10] conducted accelerated ageing experiments with cv. Cascade and Hallertauer Mittelfrüh. Ageing of 19 days resulted in a pronounced increase in caryophyllene oxide, humulene epoxide I, II and III and humulenol II levels and in the aged Hallertauer samples humulene diepoxides were detected. Interestingly, when comparing fresh with aged samples, the rate of increase of humulene epoxide II was much slower than that of its two other isomers, whilst the rate of increase of humuladienone, humulol and humulenol II was much higher than that of humulene epoxide II, suggesting that humulene epoxide II was rearranged to its secondary products. Prolonged ageing (60 days) led to dramatic losses of aroma compounds, which may be due to evaporation, polymerisation and other degradation processes[10]. The results of Tressl *et al.*[82] and Lam *et al.*[10] were largely confirmed by Peacock and Deinzer[17], who found an increase in the level of humulene epoxide I, humulene epoxide II, humuladienone and humulol upon storage of 5 different hop varieties. Humulene epoxide II appeared to be the major humulene oxidation product formed during storage[17].

Foster and Nickerson[104] aged 20 different hop varieties for a 6 month period, determined oxidation products, floral compounds and citrus compounds, and calculated a total hoppiness potential value based on these components. Using this value, the 20 different hop varieties were classified in four hop categories. One group (containing Hersbrucker, Tettnang, Record, Fuggle, Flisk, Eroica, Hallertau Millelfrüh, Willamette and Styrian) showed relatively low hoppiness potential when fresh but this potential increased significantly with hop ageing. It was therefore proposed that brewers desiring high levels of α-humulene and β-caryophyllene oxidation products in their beers should choose hops high in these two compounds that oxidise at a fairly steady rate. Mild ageing of these hop varieties prior brewing may be considered to enhance their contribution to the final beer flavour[104].

Above studies clearly demonstrate *de novo* formation of sesquiterpene oxidation products by chemical oxidation of sesquiterpene hydrocarbons during storage of hops.

1.5.2 Modifications during wort boiling and lab scale boiling experiments

During the brewing process, the hop essential oil composition of the hop products used is drastically changed. Changes in the volatile profile can be ascribed to losses and modification of hop essential oil volatiles. The wort boiling process and the fermentation process play a very important role in this regard since they alter the hop oil volatile fingerprint by chemical oxidation and biotransformation respectively. This section gives an overview of the most important modifications that occur during the wort boiling process. To generate new insights into the complex chemistry of hop oil volatiles during wort boiling, researchers have also been performing lab scale boiling experiments with pure reference compounds and hop oil fractions. The results from these experiments will also be discussed into detail.

1.5.2.1 Wort boiling

In general, high losses of hop aroma substances are observed as a result of wort boiling[20,131,132]. About 85% of the hop oil evaporates from the kettle during boiling for 90 min[133]. During wort boiling, most, if not all, of the hydrocarbons are oxidised, polymerised, or steam distilled out of the wort[28]. Directly after hop addition, the major hop oil volatiles such as β-myrcene, β-caryophyllene, and α-humulene show a very strong increase in concentration, which however diminishes quickly to a very low level at the end of the boil and, after the whirlpool, there is almost nothing left. Linalool seems to disappear even faster during boiling than the two sesquiterpenes. However, in some brews, an increase of linalool at the end of boiling or during the whirlpool rest was observed, which might be caused by hydrolysis of linalool glycosides[131]. Kishimoto *et al.*[20] studied the behaviour of hop oil volatiles upon wort boiling. Two distinct patterns of decrease of the hop oil volatiles were observed. Firstly, the levels of β-myrcene and linalool fell rapidly during the boiling process in a pattern corresponding to a quadratic curve. Secondly, a more gentle and linear decrease

was observed for β-eudesmol, α-humulene, humulene epoxide I, β-farnesene, β-caryophyllene, and geraniol. These different patterns of decrease of the hop oil compounds were explained by the difference in boiling points and solubilities of the respective substances[20]. Lam and coworkers[10] found none of the β-myrcene surviving the kettle boil and only small amounts of α-humulene and β-caryophyllene were found in boiled wort. On the other hand, considerable quantities of floral/citrus compounds survived the kettle boil, with the exception of geranyl acetate. The oxidation products of α-humulene and β-caryophyllene appeared to be well extracted into worts[10].

Basically, terpene oxidation products, formed during hop kilning and storage, are better water-soluble than their terpene hydrocarbon precursor molecules and are therefore lost to a lesser extent during brewing and subsequent fermentation[10,17–19,28,113]. It is further widely assumed that chemical oxidations of terpene hydrocarbons also occur during kettle boiling[10,16,30,39,113,134]. Several researchers have indeed found indications for such oxidation reactions. Lam and coworkers[10] detected humulene diepoxides in worts hopped with cv. Cascade and Hallertauer, that were not detected in the hops, therefore suggesting oxidation of hop-derived aroma compounds during the kettle boil. Also the α-terpineol level was much higher in the worts than in their corresponding hops, which might be due to oxidation of limonene[10]. Siebert *et al.*[135] monitored the levels of several oxygenated compounds as a function of the boiling time upon late addition of leaf hops. Although the levels of some compounds in the wort appeared erratic with time, humulol, humulenol II, humulene epoxide I and humulene diepoxides increased with increasing boiling time[135], which may support the hypothesis that sesquiterpene hydrocarbons are oxidised into oxygenated sesquiterpenoids during wort boiling. Moreover, Kishimoto and coworkers[20] found proof for oxidative degradation since the concentration of β-damascenone increased slowly upon boiling and rose dramatically thereafter during the subsequent whirlpool-processing step[20]. Nevertheless, increases in levels of sesquiterpene oxidation products have not been proven unambiguously and, as a result, the question whether or not new odourants are formed upon boiling of hop essential oil remains a matter of debate.

1.5.2.2 Lab scale oxidation and boiling experiments

Scientific insights into the impact of boiling on the volatile composition of hop essential oil are only painfully achieved. This can be attributed to the complex intrinsic composition of hop essential oil and the myriad of chemical reactions that might occur among these volatiles. Moreover, wort volatiles further complicate the picture and various compounds are present at trace levels at which they are difficult to detect. Therefore, several researchers have been conducting lab scale boiling experiments with pure reference compounds and hop oil fractions in simplified model solutions to mimic reactions that might take place during real brewing practice wort boiling. This simplified approach proved successful as many oxidation and hydrolysis products could be identified and were also detected in beer.

To investigate the volatiles formed during degradation of β-myrcene, Dieckmann and Palamand[136] subjected purified myrcene to a 48-h degradation at 65°C. The appearance of limonene, α-terpinene, γ-terpinene and terpinolene among the β-myrcene auto-oxidation products proved the ability of myrcene to cyclize to the p-menthadiene skeletal structure (yielding limonene and the terpinenes and terpinolenes via secondary rearrangements), whereas the appearances of camphene and β-pinene support the theory of a secondary cyclization involving both the endocyclic and exocyclic double bonds of limonene (see **Figure 1-12**). Oxidation in the reaction mixture was proven by the presence of α-pinene oxide and linalool oxide. The more stable secondary products (alcohols and ketones resulting from epoxide ring openings) were also detected. Linalool, citral, geraniol and nerol originated directly from air oxidation of myrcene, while α-terpineol and carvone resulted from hydration and oxidation of limonene, respectively. p-Cymene was detected as a result of a disproportionation reaction between two limonene molecules. Polymerisation products were also observed and the rate of polymerisation of myrcene proved to increase with increasing temperature[136].

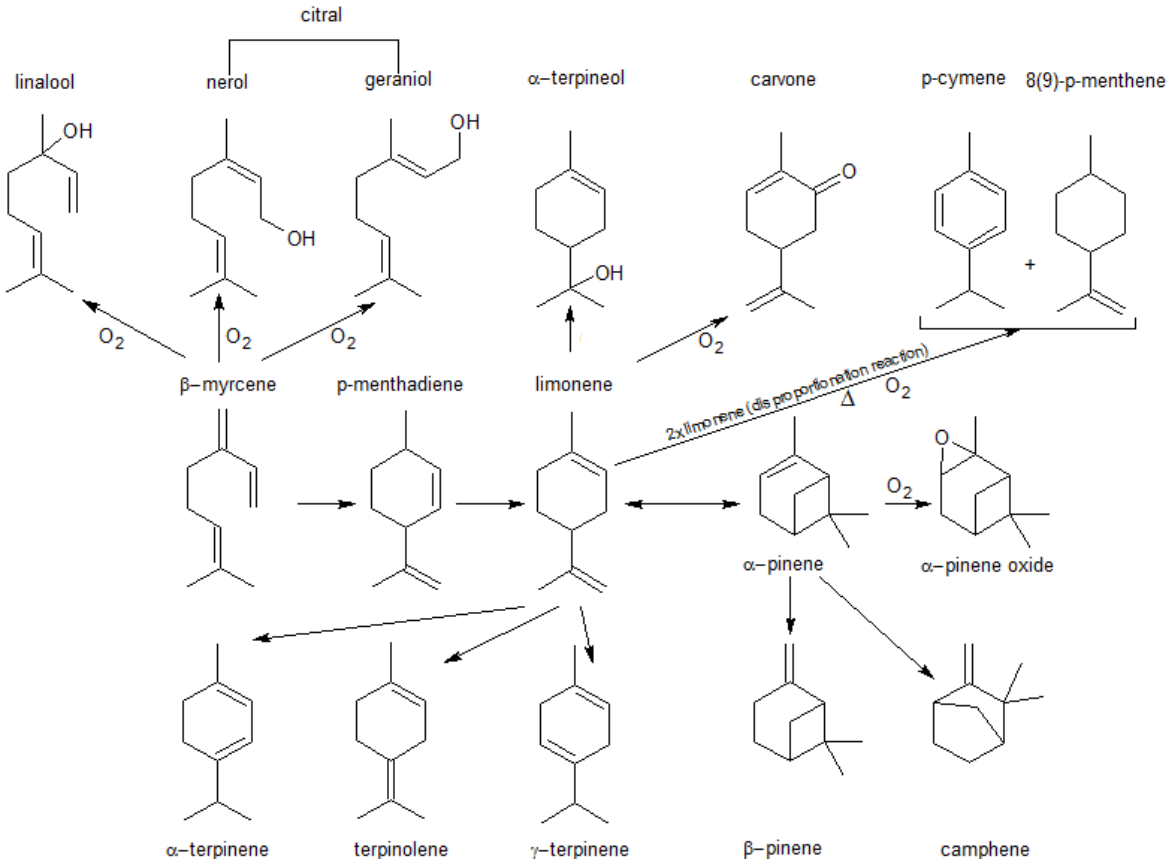


Figure 1-12. Reaction products found upon oxidation of β-myrcene[136]

Rettberg and coworkers[137] found that terpene alcohols also undergo various reactions upon reflux boiling in model solutions, as linalool, α-terpineol, geraniol and nerol were isomerised and oxidised.

Shimazu *et al.*[16] boiled pure humulene in sweet wort, yielding only a trace of humuladienone. However, when pure humulene was stored for several days much

humuladienone was formed, and either an increase in storage temperature or the presence of sunlight accelerated the oxidation of humulene[16].

Peacock and Deinzer[17] boiled pure humulene in water, which only yielded small amounts of humulene epoxide II. Boiling at pH 4.4 resulted in small amounts of humulene epoxide I, II and III and humulol, whereas humuladienone was only found after 24 hours of boiling. Boiling of caryophyllene at pH 4.4 yielded caryophyllene oxide and caryolan-1-ol[17].

Humulene diepoxide A, when boiled at pH 4, produced more than ten hydrolysis products, among them diastereomeric humuladiene triols, an exo-methylene diol, a saturated tricyclic triol and a bicyclic triol with an exo-methylene group (see **Figure 1-13**)[27].

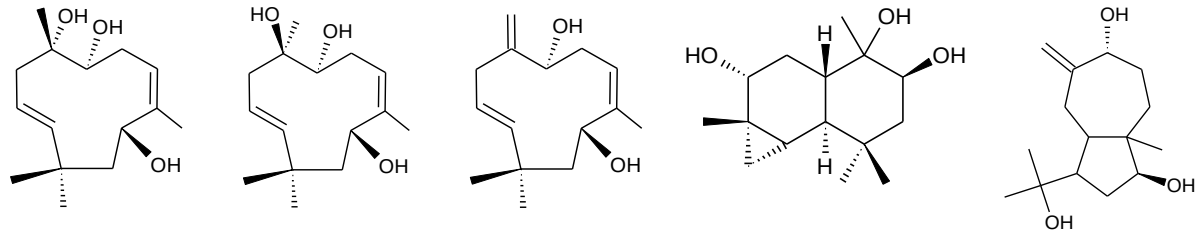


Figure 1-13. Humulene diepoxides A hydrolysis products. Diastereomeric humuladiene triols (1,2), exo-methylene diol (3), saturated tricyclic triol (4), bicyclic triol with exo-methylene group (5).

Yang and Deinzer[138] reported on the lab scale hydrolysis of humulene epoxide II and III. The compounds identified from hydrolysis of humulene epoxide II were also found among the hydrolysis products of humulene epoxide III, due to a reversible transformation between the two epoxides proceeding through a bicyclic diol (see **Figure 1-14**, **(1)**). Humuladienone, humulenol II, tricyclohumuladiol **(2)**, a vicinal diol **(3)** and many other alcohols were found among the hydrolysis products. The authors underlined that humulene epoxide I is more resistant to hydrolysis and does not result in the same products[138]. In a follow-up study, Yang *et al.*[84] only detected humulenol II and another humulene allylic alcohol **(4)**, next to humulene epoxide II and III, in hop oil. On the other hand, all humulene epoxide hydrolysis products formed upon hydrolysis of humulene epoxide II and III were detected in beer, except for the hydrolysis products only formed by humulene epoxide III[84]. These observations further support the general view that the brewing process may generate new volatiles.

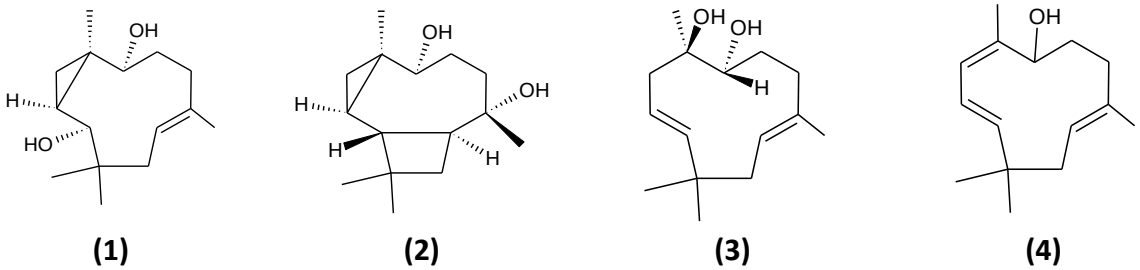
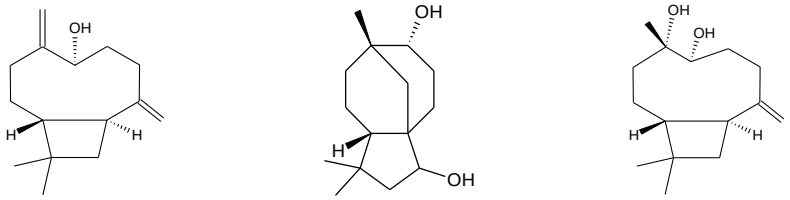


Figure 1-14. Humulene epoxide derivatives. Bicyclic diol (1), tricyclohumuladiol (2), vicinal diol (3), allylic alcohol (4).

In an analogous experiment[85], caryophyllene oxide was hydrolysed into 4S-dihydrocaryophyllene-5-one, 6(5→4)-abeo-caryophyll-8(13)-en-5-al, caryophylla-

4(12),8(13)diene-5α-ol (caryophylladienol) (see **Figure 1-15**, **(1)**), 3Z-caryophylla-3,8(13)-diene-5α-ol (caryophyllenol I), clovanediol **(2)** and a vicinal diol **(3)**. The ketone and aldehyde, which are caryophyllene oxide rearrangement products, and the allylic alcohols, formed by epoxide ring opening and elimination, were detected in hop oil, whereas clovanediol was the only product detected in beer. Caryophyllene oxide levels in hop pellets were relatively high but the epoxide was not detected in beer and it was therefore suggested that it must undergo hydrolysis and produce clovanediol during the brewing process[85]. Finally, Fukuoka and Kowaka[139] and Siebert *et al.*[135] boiled hop oil fractions but focussed more on sensory aspects related to hoppy aroma in beer. Therefore, these studies will be discussed later on.



(1) **(2)** **(3)**

Figure 1-15. Caryophyllene epoxide derivatives. Caryophylla-4(12),8(13)-diene-5α-ol (1), clovanediol (2), vicinal diol (3).

1.5.3 Modifications by yeast during fermentation

In this section, a summary of currently known biotransformations of hop oil-derived substances by *Saccharomyces cerevisiae* during wort fermentation is given. For a more comprehensive overview, we refer to Praet *et al*.[140]

1.5.3.1 Mono- and sesquiterpene hydrocarbons

The most abundant terpene hydrocarbons in hop essential oil, *i.e.* the monoterpene β-myrcene and the sesquiterpenes α-humulene and β-caryophyllene are not transformed by the yeast[141], and, are almost completely removed during fermentation of hopped wort by adsorption to the hydrophobic yeast cells[10,39,131,141,142] and migration to the foam layer[113]. Oxygenated derivatives (*e.g.* humulene and caryophyllene epoxides and alcohols) have a much higher probability to remain in the final beer and were therefore proposed as potential contributors to the hoppy aroma of beer[141].

1.5.3.2 Sulfur compounds

Terpenes containing heterocyclic sulfur atoms (*e.g.* myrcene disulfide), can undergo ring opening resulting from the reducing activity of the yeast. This reaction leads to the formation of more aromatic thiols[143]. Recently, evidence was found for the presence of S-cysteine conjugated thiols in hops. These thiols might be released by the β-lyase activity of the yeast, which cleaves the carbon-sulfur bound of the S-cysteine conjugate. Because of their low threshold-values, thiols often contribute to hop flavour in beer[100–102].

1.5.3.3 Oxygenated compounds

Carbonyl compounds and esters

Carbonyl compounds can be reduced by the yeast to alcohols[10,144]. For example, methyl ketones are partially reduced to the corresponding secondary alcohols[39]. Dehydrogenases and reductases are the key enzymes, catalyzing the reduction of a carbonyl group into a hydroxyl group[145]. Esters can be hydrolysed[10,17] or converted into ethyl esters[146] by the yeast. Methyl esters (especially the saturated ones), for example, can undergo both hydrolysis and transesterification into acids and ethyl esters, respectively[39]. Geranyl esters, largely found in the hop variety Cascade, can be hydrolysed into geraniol during fermentation[17]. Methyl esters of some conjugated acids, *e.g.* methyl geranate, can however resist hydrolysis and are therefore detectable in beer[147]. Moir *et al.*[91] demonstrated the contribution of yeast to the presence of esters and terpene alcohols in beer by transesterification during the fermentation process. The study of King and Dickinson[141], performed in model solutions, revealed that acetate esters of geraniol and citronellol are formed by lager yeast, but not by ale yeast[141].

Monoterpene alcohols

Terpene alcohols are not believed to transform spontaneously. However, they may undergo biotransformations as reported by King and Dickinson[148]. **Figure 1-16** summarises the biotransformation reactions catalysed by the yeast, consisting of reductions (geraniol to citronellol), translocations (geraniol and nerol to linalool), acetylation and cyclizations (nerol and linalool to α-terpineol) [141,148]. According to Takoi *et al.*[14], linalool, geraniol and α-terpineol levels gradually decreased during fermentation, whereas nerol was found to gradually increase during fermentation. Citronellol increased gently throughout the fermentation period, which was partially explained by the release of geraniol from glycosides[14,149].

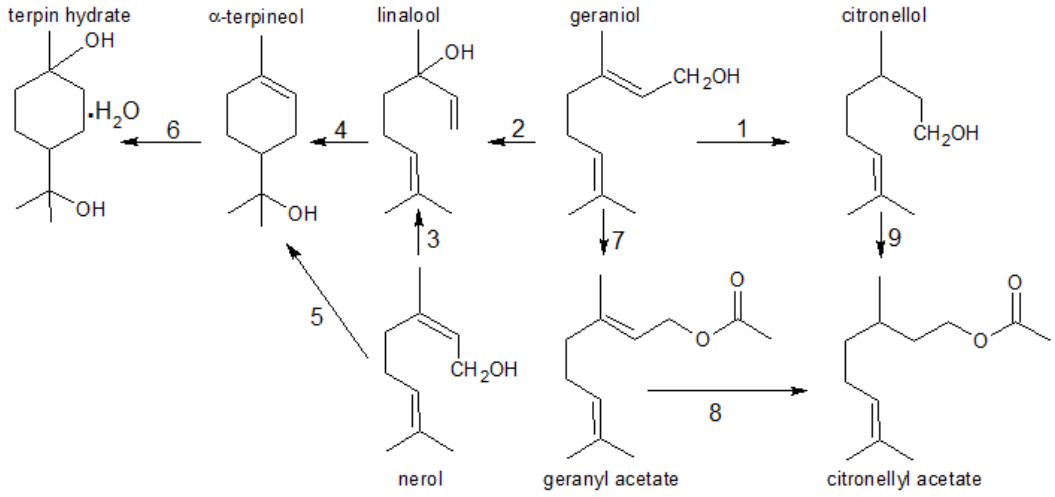


Figure 1-16. Monoterpenoid biotransformation reactions catalysed by *S. cerevisiae* (according to King and Dickinson[141,148]). Reduction (1,8), translocation (2,3), cyclization (4,5), hydratation (6), acetylation (7,9).

Sesquiterpenoids

So far, direct bioconversions of sesquiterpenoids as precursors to target compounds mediated by *Saccharomyces cerevisiae* have seldom been found in the literature[145]. However, humulol has been proposed to be a yeast reduction product of humulene epoxide II as it was not detected in model studies on the hydrolysis of humulene epoxides, but was produced in model fermentation studies of humulene[28].

Norisoprenoids

Norisoprenoids are degradation products from carotenoids. The highly flavour-active compounds β-ionone and β-damascenone, have been identified in hop oil and are present in beer at levels, at which these compounds may be important contributors to hoppy aroma of beer[147]. Lloyd *et al.*[150] observed an increase in β-damascenone levels during fermentation of wine. Also Kishimoto *et al.*[20] revealed a dramatic increase of the β-damascenone content during the fermentation of beer.

1.5.3.4 Glycosidically bound aroma precursors

Depending on the hop variety, considerable amounts of glycosides of odour active terpene alcohols and nor-carotenoids are present in hop pellets and ethanol extracts. The release of the aglycons (for examples, see **Figure 1-17**) during the brewing process could contribute to the hoppy flavour of beer[21,151,152]. Biendl *et al.*[151] and Kollmannsberger *et al.*[153] investigated the glycoside content of a lager beer, hopped with ethanol extract and hop pellets. Both acid and enzymatic hydrolysis of the glycosidic fraction of this beer led to the release of a whole series of compounds such as aliphatic alcohols, aromatic compounds, monoterpene alcohols and norisoprenoids[153]. Released aglycons with an impact on the aroma of beer could be linalool (odour threshold R-form in beer: 2.2 ppb) and β-damascenone (odour threshold in beer: 150 ppb)[22,154]. **Figure 1-17** shows some examples of aroma compounds that can occur in a glycosidically bound state.

Daenen[22] investigated the glucosidase activity of different yeast strains in more detail. It was found that only a few *Saccharomyces* strains expressed a real 1,4-β-glucosidase activity. Especially a strain depending exo-1,3-β-glucanase would be responsible for a limited glycoside hydrolysis. A more pronounced β-glucosidase activity was found in non-*Saccharomyces* yeast cells, like *Brettanomyces custersii*, isolated from fermenting Lambik[22].

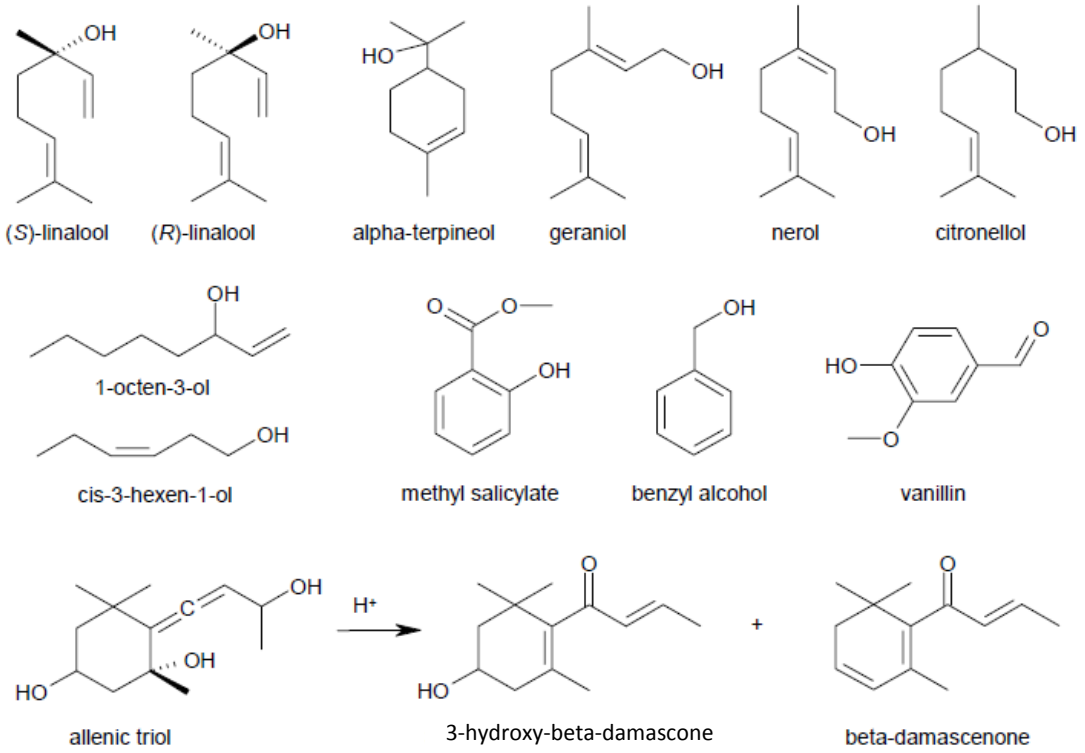


Figure 1-17. Examples of glycosidically bound aroma compounds. β-damascenone is not found as glycosidically bound compound, but can be derived from glycoconjugated precursors after acid catalysed conversion[22].

1.6 Hoppy aroma in beer

1.6.1 What is hoppy flavour?

Hoppy aroma, a hop oil-derived flavour characteristic of beer, is an essential quality criterion, especially in lager beer. The contribution of hop essential oil volatiles to beer aroma has long been questioned[90,155,156] and it was even doubted whether hopped lager beers contain a hoppy note at all[90]. Moreover, several experienced brewers maintained that lager beer has a fermentation smell and that hops have nothing to do with this. Traces of hop oil were occasionally detected in beer but these traces did not seem to contribute to hoppy aroma. At that time, there was a growing tendency to attribute hoppy smell of lager beer to the water-soluble degradation compounds of the hop bitter acids, which are formed during hop storage or during boiling of hopped wort[90]. Differences in the beer flavour when using different hop cultivars were ascribed to differing ratios of the bittering compounds or to water-soluble decomposition products (*e.g.* 2-methyl-3-buten-2-ol, 3-methylbutan-2-one) of the resin fraction[56,157]. However, at present, it is generally accepted that hoppy aroma of beer is derived from constituents present in the hop essential oil.

In the flavour wheel (see **Figure 1-2**), 'hoppy' is found under the 'aromatic, fragrant, fruity, floral' class I. It describes a fresh hop-derived aroma and does not include 'bitterness',

neither stale hop aroma[40]. Furthermore, the term 'hoppy' can be subdivided into 3 categories:

- 'hop oil' aroma, which is the flavour imparted by addition of distilled hop oil. This is also described as 'hop' aroma or the aroma of raw hops.
- 'Dry-hop' aroma or the flavour imparted by dry hops added in the tank or cask. This flavour type is reminiscent of 'hop aroma'.
- 'Kettle hop' aroma or flavour imparted by aroma hops boiled in the kettle.

Clearly, the aroma is characterised by the process used to produce 'hoppy' aroma and doesn't describe the aroma itself. Moreover, no reference standard exists for 'hoppy' aroma, reflecting the complexity of the assessment of this flavour[25].

Throughout this PhD study, the term **'hoppy' aroma** will be used to indicate the hop-derived aroma in the final beer, imparted by **conventional aromatisation** (*i.e.* hop cones or pellets added to the boiling kettle). As opposed to 'hop oil' aroma and 'dry-hop' aroma, the resulting flavour characteristic is clearly completely different from the aroma of raw hops[26,28,158–161].

1.6.2 Hopping practices and impact on hop-derived flavour in beer

Nowadays, both researchers and brewers agree that many parameters, such as hop variety, growing region, hop product and hopping regime, influence hop flavour in beer. Especially the point of time of hop addition is decisive in this regard[20,162–164].

1.6.2.1 Dry hopping

Traditionally, ales are dry-hopped, that is left in contact during conditioning with hops of a variety noted for fine aroma. By means of this method the brewer ensures survival of hop oil constituents into his finished beers in order to exhibit the desired hop character[39]. Dry hopping directly transfers the hop oil volatiles into the beer[30]. Therefore, many of the processes described in **section 1.5** operate on a much more limited scale (if at all) in the production of dry-hopped beers, compared to addition of hop products to the kettle[39,158]. Consequently, 'dry hop' aroma is very similar to the aroma of hops themselves but does not last long with beer ageing[158]. Dry-hopped beers are scored high for flavour terms such as spicy and resinous. Seaton and coworkers[165] have suggested that resinous characteristics are due to terpene hydrocarbons, whilst spicy notes are due to hop ketones, and fruity-citrus notes are due to hop esters[165].

1.6.2.2 Kettle hopping

The term 'kettle hopping' includes both 'early' and 'late' hop additions to the kettle. In conventional brewing practice, brewers add bitter hops, pellets or non-isomerised extracts

at the onset of boiling (*i.e.* 'early' hop addition) to provide the bulk of the α-acids, which than get sufficient time to become isomerised to the bitter tasting iso-α-acids. Since hop essential oil volatiles are largely lost by stripping during wort boiling, brewers have been adding hop pellets about 10 min. before kettle knock-out (*i.e.* 'late' hop addition) to impart an intense hoppy aroma to their beers. This 'late hop' aroma is typically characterised by a floral/citrusy bouquet. Traditionally, classic varieties are added to impart this flavour attribute, and, sometimes these hops are even added at the stage of wort clarification[158]. Nowadays, less traditional varieties (*e.g*. Cascade) are also added to impart late hop aroma.
However, **addition of aroma hops at the onset of boiling** is in some cases also performed since this hopping practice would impart a much more refined **'spicy/herbal'** note to the final beer[25,27,158]. In this regard, **'kettle hop' flavour** has been defined as the hop-derived flavour of beer, obtained by boiling of hop cones or pellets and subsequent fermentation[23]. Other terms such as **'European'** or **'noble' kettle hop aroma** are often found in literature[25–28]. They are linked to the use of European aroma varieties such as **Saaz, Spalt, Hallertau and Tettnang** which are believed to contribute to a 'noble hop aroma'[25]. Especially 'noble' kettle hop aroma which is thus obtained upon vigorous boiling of 'noble/European' aroma hops, has been associated with 'spicy/herbal' and 'fragrant' notes[27]. Researchers and brewers agree that a fine and balanced 'noble kettle hop' aroma is an essential quality characteristic of lager beer. Especially for traditional Pilsner-type beers, usually produced with higher amounts of hops compared to lager beer[166] , a fine hoppy aroma can be regarded as 'the soul' of the beer[167]. This delicate aroma is said to be fleeting in beer, decreasing as it ages, but it is the ultimate goal in making a traditional European-style lager or pilsner[158].
Many researchers have been using the term 'kettle hop' aroma to indicate the aroma that originates from 'early' hop addition, and, most of the time, in case of 'late' hop aroma, this is explicitly specified. Also throughout this PhD manuscript 'kettle hop' aroma will indicate the aroma derived from 'early' addition of aroma hops, whereas in case of 'late' hopping, the term 'late (kettle) hop' aroma will be used.

The difference between the aroma of raw hops and kettle hop aroma is not surprising, bearing in mind that hop oil constituents will be subjected to different physical, chemical and biochemical processes during beer production[91]. Hop oil components will be exposed to a period of boiling with some evaporation and some compounds will undergo chemical transformations. Furthermore, during fermentation, hop oil volatiles are lost by entrainment with the gaseous carbon dioxide, nonpolar substances are lost by adsorption to yeast, and several compounds can be metabolised. Finally, nonpolar compounds are further lost by adsorption to filter media[39]. As a result, 'kettle hop' aroma is much more complicated than 'dry hop' aroma.

1.6.3 Odour impact compounds of hoppy aroma

1.6.3.1 Role of oxygenated compounds

The work of Tressl *et al.*[88] represented a real breakthrough as, for the first time, these authors were able to detect and partly identify about 50 hop oil-derived components in a German kettle-hopped lager. Since then, as a result of intensive research, many compounds have been proposed to be responsible for, or at least connected to hoppy aroma of beer. In general, the nonpolar terpene hydrocarbon fraction is not considered to be responsible for hoppy flavours in kettle hopped beers[17,88,168]. Buttery and Ling[169] suggested that rather the volatile, water soluble compounds would be the basic principles responsible for hoppy aroma of beer. Kuroiwa *et al.*[157] speculated that these volatile compounds would be formed by oxidation of terpenoids during aging of hops and during the boiling process in the kettle, and thus would contribute to hoppy aroma. Indeed, oxidation products, which are better water-soluble and therefore have higher chances to survive the brewing process, are commonly found in beer and have been suggested to contribute to hoppy aroma by many other researchers[12,17,18,20,28,78,139].

1.6.3.2 Floral/citrusy note

If a floral and citrusy aroma is detected in beer, than compounds such as linalool, geraniol, citronellol, and, to a lower extent, 2-nonanol or 2-undecanol are probably involved[25]. Many authors pointed out the role of linalool for a floral aroma. Lam and coworkers[10] showed the relation between floral/citrus compounds (linalool, geraniol, citronellol) and a floral/citrus aroma in beer[10]. Seaton *et al.*[165] found a correlation between the level of linalool and the degree of hop flavour for a series of late-hopped and dry-hopped beers. Kaltner *et al.*[132,170,171], Kaltner and Mitter[134] and Fritsch and Schieberle[166] even reported on linalool as a marker for prediction of the intensity of hoppy aroma and this particular volatile would therefore be a good quality indicator of hopped beers[172]. However, according to Peacock [158], linalool is quickly steam distilled out of the wort during kettle boiling and it is therefore not a suitable indicator for 'kettle hop' aroma. Linalool is probably an important contributor to 'late hop' aroma, although other compounds contribute as well, and it appears to be only a minor contributor to 'dry hop' aroma in beer[158]. Next to linalool, geraniol, α-terpineol[10,77,139], β-ionone[12,77], citronellol[10,26,77,149] and geranyl acetate[77] have been associated to the floral aspect of hoppy aroma. However, in the end only the linalool concentration appears to be above its threshold in beer[11,170,171]. Nevertheless, other components might eventually contribute through additive/synergetic effects. Takoi *et al.*[149] found that linalool interacts with geraniol and β-citronellol, whose concentration depends on the yeast metabolism. According to Peacock and coworkers[173], geranyl esters are found in large amounts in the hop

variety Cascade and they can be hydrolysed into geraniol during fermentation, which would explain the typical floral aroma of beers hopped with this variety[173]. In dry-hopped beers, citral aldehydes could participate to the citrus aroma[10].

1.6.3.3 Spicy/herbal note

The 'spicy/herbal' flavour note of kettle hopped beer has been related to oxygenated sesquiterpenoids[16,17,19]. Researchers have in particular been focussing on α-humulene and β-caryophyllene oxidation and hydrolysis products[10,17,18,28,84,85,138]. For example, the humulene derivatives humuladienone, humulene epoxide I, II, III, humulenol II, humulol, and humulene diepoxides A–D (see **Figure 1-18**, **(1)**,**(2)** and **(3)**) were all, at a particular time, proposed to be important for kettle hop aroma[10,11,16,17,20,28,77,113]. At first, the primary oxidation products, in particular humulene epoxides, were proposed as important contributors[10,17,173]. Next, researchers focused on humulene epoxide and caryophyllene oxide hydrolysis products[84,85,138,174]. Yang and Deinzer showed that under hydrolytic conditions, humulene epoxides and caryophyllene oxide could undergo hydrolysis and isomerisation reactions and produce a large number of compounds[84,138]. Some of these compounds were found in beer and could be above their flavour threshold values in beer. Complex aromas with citrus, tropical, fruity, woody, spicy and floral notes were recorded. The authors summarised the aroma characteristics of the hydrolysis products of humulene epoxide II and III as 'citrus', 'tropical fruit', 'woody', 'spicy' and 'floral'[84]. Mixtures of hydrolysis products of caryophyllene epoxide were described as 'pine', 'cedar', 'spicy', 'rubber', 'floral', 'herbal', 'pineapple' and also slightly 'lime'[85].

However, several researchers questioned the contribution of these volatiles to kettle hop aroma[11,26,83,139]. Various researchers reported that the flavour of (individual) oxygenated sesquiterpenoids and their derivatives do not match spicy or hoppy character[28,43,83,139]. Moreover, according to Irwin[83], neither of the above-mentioned compounds appears to reach its flavour threshold value. In a study using the GC-O technique on beer, Sanchez *et al*.[26] were unable to detect aroma in the region of elution of the humulene epoxides and diepoxides. Lermusieau *et al.*[120] and Lermusieau and Collin[7] identified hop aromatic compounds in beer using GC-O. They concluded that linalool, DMTS, 2-methyl-3-furanthiol (see **Figure 1-18**, **(4)**), β-damascenone, γ-nonalactone **(5)**, humuladienone, ethyl cinnamate **(6)**, as well as a number of unidentified components, are important contributors to the flavour of hopped beers. Moreover, they stated that earlier mentioned potential hop character impact compounds such as terpinen-4-ol **(7)**, α-terpineol, citronellol, geraniol, humulenol II, humulene epoxide I and II, linalool oxide and β-ionone probably do not influence the hoppy character of beer. In a more recent study, Fritsch and Schieberle[166] found linalool as one of the most important character impact odourants in a Bavarian

Pilsner-type beer and they also reported on the high odour-activity of ethyl 4-methylpentanoate. Remarkably, these authors could not find any evidence for the odour-activity of other hop oil-derived constituents, such as the humulene and caryophyllene oxidation products[166]. According to Irwin[83] and Lermusieau *et al.*[11] oxygenated sesquiterpenes can at most contribute to a variety-dependant top note.

Nevertheless, these observations do not exclude that humulene epoxides and related compounds are collectively involved to impart the spicy flavour attribute. Peppard *et al.*[160] found good correlations between increasing levels of humulene epoxides and spicy hop character in beer. Deinzer and Yang[28] fractionated hop oil (cv. Hallertauer Mittelfrüh) leading to various fractions comprising humulene and caryophyllene derived alcohols. These fractions were scored relatively high for European hop aroma. Goiris and coworkers[19] enriched the sesquiterpenoid hop oil fraction of the varieties Tettnanger, Hersbrucker, Saaz, Spalter Select and Perle. Upon addition of these fractions to non-aromatised iso-α-acid bittered lager beer, a spicy/herbal flavour note, reminiscent of typical 'noble' hop aroma was observed. In addition, Van Opstaele *et al.*[175] reported on a flavour threshold value of isolated oxygenated sesquiterpenoid fractions in beer of 10–20 ppb, pointing to the high flavouring potential of these particular compounds. Clearly, there is a correlation between the presence of oxygenated sesquiterpenoids and 'spicy/herbal' flavour attributes in lager beer. A good correlation does, however, not necessarily reflect a cause-and-effect relationship.

Although the relevance of humulene and caryophyllene oxidation products was seriously doubted, recent research again points in the direction of oxygenated sesquiterpenoids as key impact flavour compounds for kettle hop aroma. Researchers have been claiming that there are still minor flavour-active sesquiterpenoids that yet have to be identified[12,19,114,124,139,146]. Already in the 80's, Fukuoka and Kowaka[139] stated that humulene epoxide II or humulenol II are not the key components of herbal flavour, but instead two unidentified oxygenated sesquiterpenoids were pointed out. Also Goiris *et al.*[19] proposed in their study on the isolated hop sesquiterpenoid fraction that there had to be yet unidentified flavour-active compounds responsible for hop-derived herbal/spicy flavour. In 2006, Kishimoto *et al.*[12] found three 'spicy' compounds in the oxygenated sesquiterpenoid fraction of hops via GC-O, but they were still not able to identify these compounds. During the last decade, several oxygenated sesquiterpenoids were proven to be flavour-active. In 2007, Eyres *et al*.[114] identified 14-hydroxy-β-caryophyllene as a strong 'cedarwood' odourant in the spicy fraction of hops and, two years later, Nielsen[146] identified caryohylla-3,8(13)-diene-5β-ol (see **Figure 1-18**, **(8)**) as the key odour impact compound responsible for the spicy/woody kettle hop aroma in ales. Both authors[114,146] also mentioned the presence of

other still unidentified minor odour-active 'cedarwood' compounds in the spicy fraction of hop essential oil.

Clearly, oxygenated sesquiterpenoids are related to kettle hop aroma. In this respect, brewers may even store their hops under conditions that promote some mild oxidation, since beers brewed with such hops tend to produce more hoppy aroma in the final beer[10,104,113]. Analogously, the kettle boil might also generate sesquiterpene oxidation products and therefore enhance 'spicy/herbal' flavour in beer. Fukuoka and Kowaka[139] boiled a hop oil fraction expressing weak herbal flavour and further fractionated this boiled hop oil fraction. They found a fraction exhibiting herbal/spicy flavour without floral flavour and concluded that the impact flavour compounds which impart herbal flavour are some unidentified sesquiterpenoids, which were also present in domestic beer[139]. Siebert *et al.*[135] isolated the hop essential oil fraction from hops and brewed a beer with both the neutral fraction and the heated neutral fraction to test the hypothesis that heating of material might be needed to produce some hoppy flavour. The taste panel responses to the two beers were very similar. However, the beer brewed with the unheated neutral fraction was perceived as greater in intensity whereas the beer made with the heated neutral fraction was rated higher in herbal[135]. In conclusion, vigorous boiling of hops might be of utmost importance for the development of kettle hop aroma in beer.

1.6.3.4 Other contributors to kettle hop aroma

Besides the monoterpene alcohols (linalool, geraniol, citronellol, α-terpineol) and sesquiterpene oxidation products discussed above, much more compounds were considered in literature as contributors to kettle hop aroma. Oxygenated sesquiterpenoids such as τ-cadinol[77,139] and α-eudesmol[77] (see **Figure 1-18**, **(9)**) were proposed to be important in respect to kettle hop aroma. In addition, typical esters (ethyl 2-methylbutanoate, ethyl 4-methylpentanoate, ethyl 3-methylbutanoate, ethyl 2-methylpropanoate), esters of linalool and geraniol (*e.g.* geranyl acetate), and some uncommon cyclic ethers, such as karahana ether and hop ether are often mentioned as key contributors to the floral hop-derived aroma in beer[12,17,88]. Also linalool oxide[77,113] has been proposed as a contributor to kettle hop aroma and rose oxide **(10)**, a potent floral and herbaceous odourant, was found to be distinctive for an ale hopped with cv. Centennial[146]. The oxidative carotene degradation products β-ionone and β-damascenone exhibit extremely low flavour thresholds and might also contribute to kettle hop aroma[88]. In addition, sulfur compounds, which have somehow received less attention, have been proposed as important contributors to beer aroma. Lermusieau *et al.*[11] reported on dimethyl disulfide, dimethyl trisulfide, diethyl disulfide and 2-methyl-3-furanethiol. Moreover, 4-mercapto-4-methylpentan-2-one[98,142] and, 3-sulfanyl-4-methylpentan-1-ol and 3-sulfanyl-4-methylpentyl acetate[99,127] have been identified as main contributors to the characteristic strong fruity/black currant of beers hopped with US

cultivars and to the specific exotic fruity-like/white wine-like flavours of beers brewed with the hop cultivar Nelson Sauvin, respectively.

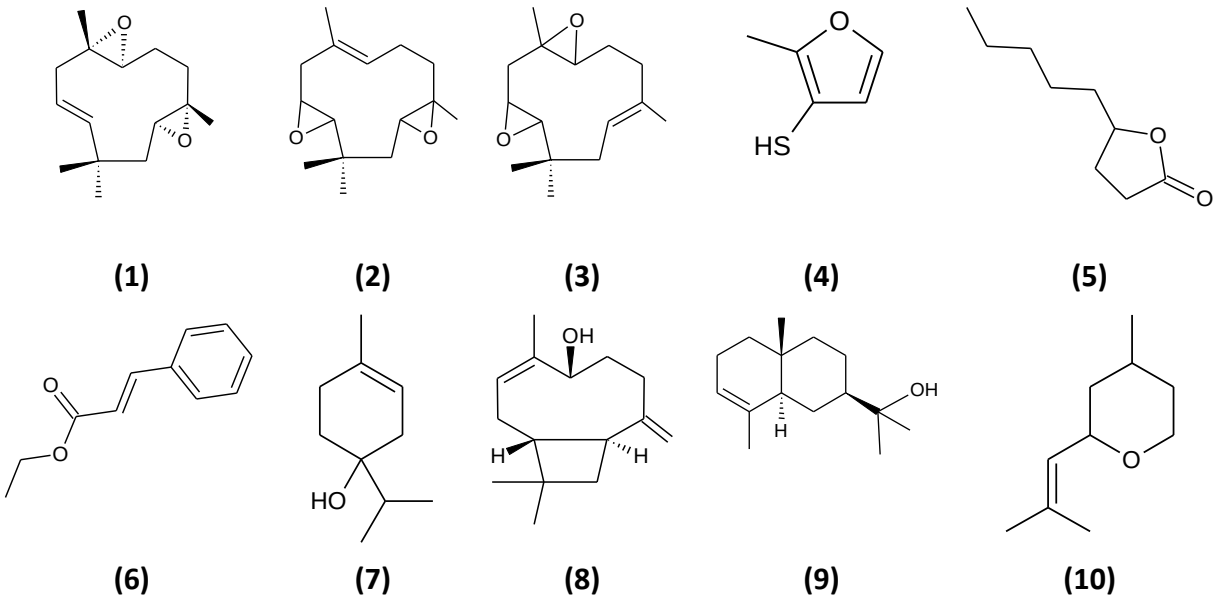


Figure 1-18. Contributors to hoppy flavour in beer. Humulene diepoxides B (1), C (2) and D (3), 2-methyl-3-furanthiol (4), γ-nonalactone (5), ethyl cinnamate (6), terpinen-4-ol (7), caryohylla-3,8(13)-diene-5β-ol (8), α-eudesmol (9), rose oxide (10).

1.6.4 Concluding remarks

Clearly, of all the currently identified sesquiterpenoids, not a single individual component has been spotted that exhibits kettle hop aroma. While hop-derived bitterness is dominated by a few compounds, hoppy aroma is the result of a great many substances[65] and the 'hoppy' flavour impression is probably the result of additive, synergetic and/or antagonistic effects among different compounds[7,8,14,113]. Moreover, lack of oxygenated sesquiterpenoid reference compounds and of high quality mass spectra hamper adequate identification[114,124]. As a result, the chemical and sensorial background of kettle hop aroma is still largely unknown[5,26]. Until today, hoppy aroma remains a highly controversial topic that still requires investigation in depth by application of state-of-the-art aroma technology[15].

Chapter 2

LAB SCALE BOILING OF HOP ESSENTIAL OIL (CV. SAAZ) IN AQUEOUS MODEL SOLUTIONS

Chapter 2 corresponds to:

Praet, T.; Van Opstaele, F.; Steenackers, B.; De Brabanter, J.; De Vos, D.; Aerts, G. and De Cooman, L.

Changes in the hop-derived volatile profile upon lab scale boiling.

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Chemical-analytical profiling of unboiled and lab scale boiled hop essential oils (cv. Saaz) in aqueous solutions for determination of quantitative and qualitative changes in the hop oil-derived volatile profile upon boiling. Sensory evaluation of boiled hop essential oil by addition to non-aromatised iso-α-acid bittered lager beer.

Tatiana Praet and Jelle Dendooven (bachelor student) performed the experiments. Dr. Bart Steenackers prepared the mixtures of oxygenated sesquiterpenoid reference compounds. The final manuscript was written by Tatiana Praet and revised and adapted after critical input by Prof. Luc De Cooman and Dr. Filip Van Opstaele.

2 LAB SCALE BOILING OF HOP ESSENTIAL OIL (CV. SAAZ) IN AQUEOUS MODEL SOLUTIONS

2.1 Introduction

Despite the increasing popularity of strongly hopped ale beers, lager beers remain most widely consumed, which can be largely attributed to their excellent thirst-quenching qualities and a refined, well-balanced hop-derived aroma. This so-called 'kettle hop' or 'hoppy' aroma is achieved by kettle hopping[25,28,113] and comprises flavour characteristics that are clearly different from the added hops[26,28,159]. By GC-MS and GC-O analysis of hops, hopped and unhopped beers, numerous researchers have already investigated the nature of the hop-derived volatile profile of hops and beers in an attempt to pinpoint the compounds that impart the highly desired 'kettle hoppy' aroma[7,11,20,26,159,166,176]. However, because of the intricate chemical composition of hop essential oil, the interference of malt- and fermentation-derived peaks in the chromatograms[16], the direct comparison of hops with beer without taking intermediate samples into consideration, and potential synergetic/additive effects that are not detectable with GC-O[113], determining analytical and sensorial changes in the hop-derived volatile profile that actually impact 'hoppy' aroma of beer appears to be illusive.

The general opinion is that hop oil oxidation products play a key role in 'kettle hop' or 'hoppy' aroma since these compounds, formed during hop kilning and storage, are better water-soluble than their terpene hydrocarbon precursor molecules and are therefore lost to a lesser extent during brewing and subsequent fermentation[10,17–19,28,113]. Indeed, most of the hop-derived compounds found in beer are oxygenated terpenoids[17,28,113]. It is further widely assumed that chemical oxidations of terpene hydrocarbons also occur during kettle boiling[10,16,30,39,113,134] and that these changes in the hop-derived volatile profile would be a major cause for flavour differences between 'dry' and 'kettle' 'hoppy' aroma. Several investigations have proven that the sesquiterpene hydrocarbons (SHCs) β-caryophyllene and α-humulene, amply present in aroma hop varieties, may indeed be oxidised upon boiling in model solutions and that oxidation products may be further hydrolysed into a series of alcohols[16,17,27,28,84,85,138,174]. Also terpene alcohols such as linalool, α-terpineol, geraniol and nerol can become isomerised and oxidised during reflux boiling in model solutions[137]. However, research involving boiling experiments with total hop essential oil or hop oil fractions has seldom been reported[135,139]. Therefore, in this chapter we aim at investigating changes in the levels of hop oil constituents and potential formation of new compounds when boiling total hop essential oil in a closed aqueous model solution. In addition, we will

assess sensory characteristics of boiled hop essential oil, spiked to non-aromatised iso-α-acid bittered lager beer.

The use of multivariate data analysis techniques will be essential to get insight in the complex GC-MS derived data blocks. Multivariate procedures in sensory research have been thoroughly reviewed[177–179] and relatively simple chemometric methods such as Partial Least Squares (PLS), Exploratory Factor analysis (EFA) and Principal Component Analysis (PCA) have been used extensively. PLS was already used in the 80's to find correlations between analytical and sensory results on hop flavour in beer[160]. FA has been used to classify beers on the basis of their sensory characteristics[180] and to discriminate hop essential oils[106]. Also factors of a dataset derived from gas chromatographic profiling of fermentation experiments, were proposed useful for classification of beers[181]. PCA has been employed to show resolution of different beer styles and close proximity of duplicate samples[182] and, recently, to estimate the key aroma compounds related to hop aroma characteristics in beer[183]. Our own research group has frequently applied PCA in order to gain insight in the discrimination between single-variety total hop oils, polar, and floral hop essences on account of their volatile composition[123,175,184]. Though PCA can depict structure and provide a rough draft of clustering in the data, cluster analysis (CA), an unsupervised pattern recognition (PARC) technique, is more adequate to find group structures and moreover, CA has been employed in brewing and hop research on both chemical and sensory data[185–187]. In contrast to the above mentioned exploratory data analysis (EDA) and modeling techniques, supervised pattern recognition, including Discriminant Analysis (DA) and Soft Independent Modeling of Class Analogies (SIMCA), has been employed to a lesser extent, but, nevertheless, proved to be a very powerful tool for classification[186,187]. In this chapter and also in the following chapters, PCA will be employed to screen data for group structures and correlations, whereas CA is used for classification of samples.

2.2 Experimental

2.2.1 Chemicals

2.2.1.1 Reference compounds

The following reference compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical grade:

2-decanone (99.5%); 2-undecanone (99.0%); 3-methylbutyl isobutanoate (≥98%); camphene (95.0%); caryophyllene oxide (≥99.0%); limonene (97.0%); linalool (98.5%); methyl 3-nonenoate (99.8%); methyl geranate, ocimene (≥90.0%, mixture of isomers); methyl heptanoate (≥99%); methyl octanoate (99.8%); nonadecane (≥99.8%); nonanal (95.0%); p-cymene (≥99.0%); terpinolene (≥90.0%); *trans*-β-farnesene (≥90%); α-humulene (≥98.0%); α-pinene (98.0%); β-caryophyllene (98.5%); β-myrcene (≥95.0%); β-pinene (99.0%); γ-terpinene (≥97.0%).

2.2.1.2 Mixtures of reference compounds

The lack of authentic oxygenated sesquiterpenoid (OS) reference compounds represents a serious problem to verify analytical data (mass spectra, retention indices) and sensory descriptions. To tackle this problem, reference mixtures containing OSs were prepared via chemical treatment of α-humulene and β-caryophyllene reference compounds. Via this approach, we were able to collect additional information about the identity of particular compounds (formed upon boiling hop-derived sesquiterpene hydrocarbons (SHCs)) by comparison of their calculated retention indices (RIs) and mass spectra with RIs and mass spectra of compounds present in the prepared reference mixtures. Epoxidation of α-humulene, β-caryophyllene and iso-caryophyllene, and subsequent acid-catalysed rearrangement of the formed epoxides, photosensitised oxidation of α-humulene and β-caryophyllene and singlet oxygenation of β-caryophyllene were carried out for the preparation of SHC-derived epoxide and alcohol mixtures.

Epoxidation of α-humulene, β-caryophyllene and iso-caryophyllene

Sesquiterpene-derived epoxides were synthesized by epoxidation with *m*-chloroperoxybenzoic acid (Sigma Aldrich, ≥77%): equimolar amounts (0.05 M) of sesquiterpene (α-humulene, β-caryophyllene or iso-caryophyllene) and *m*-chloroperoxybenzoic acid in CH_2Cl_2 were stirred for 0.5 h at 0°C. After extraction with 0.1 M $NaHCO_3$, the epoxides were isolated by removing the organic solvent under reduced atmosphere. β-Caryophyllene and iso-caryophyllene are quantitatively converted to caryophyllene oxide and isocaryophyllene epoxide respectively, whereas α-humulene is

converted into a major fraction of mono-epoxides (humulene epoxide I, II and III with a ratio of 15:75:10, based on GC-FID peak areas) and a minor fraction of diepoxides.

Acid-catalysed rearrangement of sesquiterpene epoxides

The sesquiterpene-derived epoxides were dissolved in CH_2Cl_2 and reacted in the presence of 5 wt% of strong acid (*p*-toluenesulfonic acid (Sigma Aldrich, >98.5%) or Nafion SAC-13 (Sigma Aldrich)) at 0°C for 2h. The samples were extracted with 0.1 M $NaHCO_3$, filtered and analysed by GC-MS.

Photosensitised oxidation of α-humulene and β-caryophyllene

To obtain the allylic hydroperoxides of α-humulene and β-caryophyllene, photosensitised oxygenation reactions were performed in glass vials at room temperature under 1 bar O_2. In a typical oxidation procedure, α-humulene or β-caryophyllene was added to a 0.1 mM solution of methylene blue in ethanol (EtOH) and the solution was irradiated with a 150 W halogen lamp (Schott KL 1500). Samples were reduced to their corresponding allylic alcohols with an excess of triphenylphosphine prior to analysis. Photooxygenation of α-humulene and subsequent reduction resulted in the formation of three allylic alcohols: humulenol II, humulene allylic alcohol X and humulene allylic alcohol Y (ratio 50:31:19, based on GC-FID peak areas). From β-caryophyllene, only two of the three possible isomeric allylic alcohols were formed: caryophylla-4(12),8(13)-dien-5-ol (caryophylladienol) and caryophylla-3,8(13)-dien-5-ol (caryophyllenol) (ratio 86:14, based on GC-FID peak areas). As a control, the hydroperoxides were prepared according to a second procedure, involving a molybdate catalysed disproportionation of H_2O_2, resulting in the same compounds in the same ratios. Based on HS-SPME-GC-MS peak areas, the mixture contains 73.4% caryophylla-4(12),8(13)-dien-5α/β-ol (co-elution of both isomers), 4.5% (3Z)-caryophylla-3,8(13)-dien-5α-ol and 8.6% (3Z)-caryophylla-3,8(13)-dien-5β-ol.

2.2.2 Plant material

Hop essential oil was extracted from hop pellets T90 (crop year 2011) cv. Saaz, kindly provided by the Barth-Haas Group (Joh. Barth & Sohn GmbH & Co. KG, Nürnberg, Germany). Pellets (100 g) were stored in the freezer (-18°C) to avoid oxidative degradation of hop oil compounds. Prior to extraction, 50 g pellets were disrupted using an electric coffee grinder (Krups 75) to facilitate subsequent extraction.

2.2.3 Isolation of hop essential oil from hop pellets

2.2.3.1 Steam distillation procedure

A steam distillation apparatus (Brateq, The Netherlands) was used to isolate hop essential oil from the pellets. Ground hop pellets (40 g) were transferred into a 1-L round bottom flask,

containing 500 mL MQ-water (MQ purification system, Synergy 185, Millipore S.A., Molsheim, France). The flask was heated to boiling temperature using an Electrothermal Electromantle (1 L capacity, Rochford, Essex, UK) and the distillation was carried out for 3 h, whereupon the hop essential oil was collected and diluted in ethanol (1/10 v/v hop essential oil-HPLC grade ethanol (≥ 99.8 %, VWR International, Zaventem, Belgium)). The diluted oil was poured into a dark brown screw-capped glass vial (20 mL, amber glass, Chromacol, Welwyn Garden City, UK) and stored in the freezer (-18°C) until further analysis.

2.2.3.2 Supercritical Fluid Extraction (SFE)

Hop essential oil was extracted from ground pellets, using a Dionex SFE-703 supercritical fluid extractor (Dionex, Sunnyvale, California, USA) as described by Van Opstaele and coworkers[175].

2.2.4 Boiling of hop essential oil

Hop essential oil derived from steam distillation or SFE, was diluted in MQ-water to the desired concentration (given in section 2.5) in HS-SPME vials (20 mL, clear glass, Chromacol, Welwyn Garden City, UK). The vials were closed using bimetal magnetic crimp caps containing a silicone/Teflon septum (Interscience, Louvain-la-Neuve, Belgium) and the hop oil dilution was subsequently boiled in the incubation oven of the CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland); the oven temperature was 100°C, and the stirring of samples was 250 rpm with 5 s on and 2 s off. After 1 h of boiling, the vials were removed and cooled in the cooler (3°C) of the CombiPAL autosampler.

2.2.5 Sample preparation prior to GC-MS analysis

2.2.5.1 Preparation of samples for liquid injection GC-MS analysis

In order to analyse increasing concentrations of unboiled and boiled (steam distilled) hop essential oil via liquid injection into the GC-MS, 7 unboiled (u1-u7) and 7 boiled (b1-b7) (boiled according to the procedure described in 2.4) aqueous hop oil samples (concentrations ranging from 0.8 to 14.3 g/L steam distilled hop oil) were prepared. Quantitative transfer of the hop oil compounds in these samples from MQ-water to ethanol is achieved using Solid Phase Extraction (SPE). Varian Bond Elut C18 cartridges (500 mg, 6 mL, Agilent Technologies, Lake Forest, USA) were pre-conditioned with 10 mL MQ-water, 10 mL ethanol and 10 mL ethanol/water (1/1; v/v ethanol/MQ-water), respectively. The sample (boiled or unboiled aqueous hop oil solution) was diluted with ethanol (1/1 v/v ethanol/MQ-water solution), whereupon the total content was pipetted on the C18 column and eluted. Next, the hop oil compounds adsorbed to the C18 stationary phase were desorbed by

pipetting 10 mL ethanol onto the column and collecting the eluate. The eluates were stored in the freezer (-18°C) in screw-capped brown glass vials (20 mL) until further analysis.
The SPE-derived fractions were analysed by pipetting 200 µL of the fraction and 30 µL internal standard (nonadecane, 1.508 g/L in ethanol) in a vial (clear glass, 1 mL). One µL of the solution was manually injected into the GC-MS (splitless injection; 10 µL syringe, Hamilton, Reno, USA) and analysed using fast oven programming (see **section 2.2.6**).

2.2.5.2 Preparation of samples for HS-SPME-GC-MS analysis

In order to investigate the analytical discrimination of unboiled and boiled aqueous hop essential oil solutions (cv. Saaz), eight HS-SPME vials, containing the supercritical fluid extract in MQ-water (hop oil concentration: 75 mg/L), were prepared: 4 vials remained unboiled and 4 vials were boiled according to the boiling procedure described above (**section 2.2.4**). All vials were analysed via HS-SPME-GC-MS using a split ratio of 1:10 and fast oven temperature programming (see **section 2.2.6**).

Two SPE-derived eluates (u2 and b2, C=1.67 and 1.68 g/L resp.), obtained as described in **section 2.2.5.1**, were also analysed via HS-SPME-GC-MS (n=3) for investigation of (semi) quantitative differences of the detected volatiles. The SPE-eluate was diluted in ethanol (1/10 v/v) and 5 µL of the dilution was added to 4.750 µL MQ-water, 10 µL nonadecane (C=1.508 g/L) and 235 µL ethanol in a HS-SPME vial. The samples were analysed using splitless injection and slow oven programming (see **section 2.2.6**). Calibration curves (10-point calibration curve, 0-250 µg/L) of reference compounds were drawn up by pipetting 0 to 250 µL stock solution (5 mg/L), 10 µL nonadecane and 250 to 0 µL ethanol in a HS-SPME vial (splitless injection, slow oven programming, described in **section 2.2.6**). These calibration curves were used to calculate the recoveries upon boiling (on the basis of concentrations in u2 and b2). If no reference compound was available, recoveries were calculated on the basis of the normalised peak area (by dividing the average peak area to hop oil concentration ratio of the boiled samples by the average peak area to hop oil concentration ratio of the unboiled samples).

Finally, qualitative differences between unboiled and boiled hop essential oil were investigated analytically by preparing boiled aqueous hop essential oil solutions in 4 different concentrations (10, 100, 500, and 1000 mg supercritical fluid extract/L MQ-water, respectively). These boiled hop essential oil solutions of 100, 500 and 1000 mg/L were further diluted in MQ-water to 10 mg/L for HS-SPME-GC-MS analysis, whereas a 10 mg/L boiled hop essential oil solution remained undiluted. The qualitative volatile profile of all samples was compared to the volatile fingerprint of an unboiled hop essential oil solution of 10 mg/L, using HS-SPME-GC-MS (splitless injection and slow oven programming as described in **section 2.2.6**).

2.2.6 HS-SPME GC-MS analysis of volatiles

Headspace solid-phase microextractions of hop oil preparations were automated using a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland). For SPME isolation of the volatiles, an extraction fibre with PDMS coating (100 µm) was selected. The extraction fibre was exposed into the headspace of the vial (25 mm). For a maximum detector response, an extraction temperature of 60°C and duration of 45 min were selected for further GC-MS characterisation of total hop essential oils[124].

Gas chromatographic operating conditions were as follows. SPME fibres with extracted volatiles were thermally desorbed in the heated inlet (split/splitless injector, 250°C) of the Ultra Trace gas chromatograph (Thermo Fisher Scientific, Austin, TX) for 3 min. Helium (Alphagaz 2, Air Liquide, Luik, Belgium) was used as a carrier gas at a constant flow of 1.0 mL/min. Injection was performed in the split mode (split ratio: 1/10) for 3 min at 250°C. Separation of the injected compounds was performed on a 40 m × 0.18 mm i.d. × 0.20 µm film thickness RTX-1 capillary column (Restek Corporation, Bellefonte, PA)[124]. Two different oven programs were used for separation of the volatiles: (1) fast oven programming: 3 min at 35°C, followed by a temperature increase of 6°C/min to 250°C and a hold of 5 min. (2) slow oven programming: initial temperature of 40°C, hold for 1 min, followed by a temperature increase of 10°C/min to 72°C, hold for 1 min, temperature increase of 2°C/min to 137°C, hold for 1 minute, a temperature increase of 1°C/min up to 172°C, hold of 1 min and finally an increase of 10°C/min to the final temperature of 250°C, which is maintained for 3 minutes.

Mass spectrometric detection of volatiles was performed by a Dual Stage Quadrupole MS (DSQ I, Thermo Fisher Scientific, Austin, TX) operating in the electron ionization mode (EI, 70 eV). The ion source was set at 240°C and the detection gain was 2 x 10^5 (electron multiplier voltage: 1446 V). Analyses were performed in the full scan operating mode (m/z = 40-265). The MS was programmed to detect positive ions and total scan time was 0.25 s (4.03 scans/s, scan rate: 995.8 amu/s). The detected compounds were identified by mass spectral comparison via the Xcalibur software (v.1.4 SR1, Thermo Fisher Scientific, Austin, TX), using the "NIST98" and "Flavour MS library for Xcalibur 2003" spectral libraries (Interscience, Louvain-la-Neuve, Belgium), via reference mass spectra found on the NIST website (http://webbook.nist.gov/chemistry/) and in books containing mass spectral information[188,189]. Next, retention indices (RI) from literature data were compared with the calculated retention indices of the volatiles, determined by using a homologous series of normal alkanes (C6-C19; Sigma-Aldrich, St. Louis, MO). Compounds were (tentatively) identified if there was a match for both mass spectrum and KI. If the mass spectrum and/or RI were not comparable with literature data, the compound was indicated as 'unknown'.

2.2.7 Brewing of non-aromatised iso-α-acid bittered lager beer

A reference lager beer was prepared at our pilot brewery (2 hL scale). To prevent introduction of hoppy aroma in view of reliable sensory evaluation of the flavour effects of the boiled hop oil, the reference beer was exclusively bittered by the addition of pre-isomerised hop extract. Brewing was performed as follows: 40 kg milled pilsner malt, 140 L reverse osmosis brewing water with $CaCl_2$ added (80 mg/L), pH 5.25; mashing-in scheme: 30 minutes at 63°C, 10 minutes at 72°C, 1 minute at 78°C; filtration: lauter tun; boiling for 1 h (atmospheric pressure, 8% evaporation), addition of 19.2 g/hL pre-isomerised hop extract (Botanix, UK, Kent) at end of boil (presumed utilisation: 65%, predicted concentration of iso-α-acids in final beer: 25 mg/L); original gravity: 12°P; clarification: whirlpool; lager yeast: W34/70 (Fermentis, pitching rate: 10^7 yeast cells/mL); 1 week fermentation (12°C); 2 weeks lagering (0°C); filtration: kieselguhr/cellulose sheets (1 µm); packaging: automatic filling with pre-evacuation and CO_2 flushing (6-head America monobloc filling equipment, Cimec, Italy) in brown glass bottles (25 cL) closed with conventional crown corks.

2.2.8 Sensorial evaluation of boiled hop essential oil in non-aromatised iso-α-acid bittered beer

Odour and aroma characteristics of boiled hop essential oil were evaluated via descriptive sensory analysis by our trained (using reference compounds, *e.g.* linalool, β-myrcene, nonanal, 2-undecanone, α-humulene, β-caryophyllene, caryophyllene oxide, and total hop essential oils) taste panel (12 panellists). For this purpose, iso-α-acid bittered beers were aromatised with boiled hop essential oil solutions (1000 mg/L, final concentration in beer: 1 mg/L). Additions to beer bottles was performed under nitrogen atmosphere (in absence of oxygen in an airlock closed workstation, Don Withney Scientific Limited). Beer bottles with and without (=blanks) addition of boiled hop essential oil were subsequently stored at 0°C for 24 h for equilibration of the hop oil-derived constituents in the beer matrix, prior to sensory evaluation. Two hours before sensory evaluation, the beers were taken out of the refrigerator. Each panellist was served two beer samples (with and without (=blank) boiled hop oil solution). Panellists were asked to score the intensity of pre-selected odour/aroma descriptors (malt/worty, fruity, floral, citrusy, hoppy, spicy, woody, hay/straw) on a scale ranging from 0 to 8 (0=not detectable, 8=very high intensity).

2.2.9 Multivariate data analysis

Principal component analysis (PCA) and unsupervised cluster analysis (CA) were performed on the HS-SPME-GC-MS-derived data of unboiled and boiled aqueous hop essential oil solutions. A demo of Solo 7.5 (R7.5.2) (Eigenvector Research, Inc., Manson, WA, USA) was used for this purpose. This software equips users to perform multivariate analyses and

includes the PLS Toolbox (chemometric multivariate analysis tools for use within the MATLAB® computational environment) graphical user interfaces.

Prior to descriptive sensory analysis of beers with and without addition of hop essential oil, a statistical significant difference was demonstrated by performing two independent triangular tests by a taste panel. A statistical significant difference at an α-level of 0.05 and 0.10 respectively (on the basis of BS ISO 4120:2004 standards) was found. For the descriptive tests, the score of a particular descriptor was calculated by taking the average of the scores assigned by the 12 panellists.

2.3 Results and Discussion

2.3.1 Analytical discrimination of unboiled and boiled aqueous hop essential oil solutions (cv. Saaz)

The hop oil volatile profile of 8 samples (prepared as described in **section 2.2.5.2**) was characterised by HS-SPME-GC-MS. The detected volatiles were subdivided in 'monoterpene hydrocarbons', 'floral' compounds, 'sesquiterpene hydrocarbons' (SHCs) and 'spicy' compounds, according to their chemical structure and the chromatographic region in which they elute[15]. The 'floral' compound class contains the oxygenated monoterpenoids and aliphatic and branched esters, alcohols, ketones and aldehydes, whereas the 'spicy' compound class consists of the oxygenated sesquiterpenoids (OSs) and aliphatic/branched esters, alcohols, ketones and aldehydes that elute in the same region (see **Figure 2-1**). This terminology is derived from the 'floral' and 'spicy' odour that these compound groups impart when isolated from total hop essential oil via SPE[15,184] .

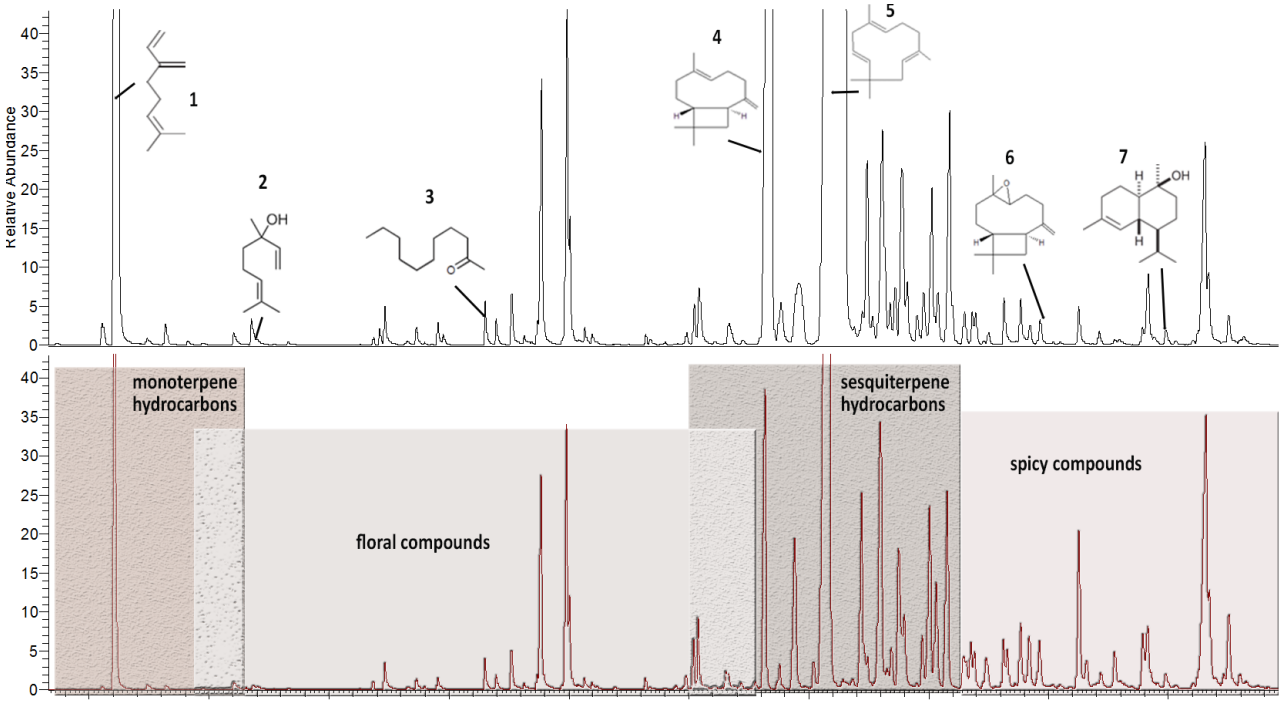


Figure 2-1. HS-SPME-GC-MS chromatogram of unboiled hop essential oil (top) and boiled hop essential oil (bottom) cv. Saaz. Classification of volatiles into monoterpene hydrocarbons (*e.g.* β-myrcene (1)), floral compounds (*e.g.* linalool (2), 2-undecanone (3)), sesquiterpene hydrocarbons (*e.g.* β-caryophyllene (4), α-humulene (5)) and spicy compounds (*e.g.* caryophyllene epoxide (6), τ-cadinol (7)) according to their structure and elution region in the chromatogram (RTX-1 column).

For each compound class, the average area ratio (peak area divided by hop oil concentration), relative area (%) and recovery (%) of the boiled samples vs. unboiled samples are summarised in **Table 2-1**. A clear decrease in the terpene hydrocarbon ratios is observed upon boiling. On the other hand, the portion of floral compounds increases from 3

% to 8 % when boiling the hop oil solutions, whereas the portion of spicy compounds increases from only 1 % up to 7 %. The recoveries of the 4 compound classes (**Table 2-1**) indicate that the increases of the area/concentration ratio of floral and spicy compounds after boiling can be attributed to an increase in their absolute level in combination with a decrease in the level of terpenes, suggesting oxidative transformation of terpenes into oxygenated derivatives.

Table 2-1. Average peak area/concentration ratio, relative area and recovery (upon boiling) for the 4 compound classes of unboiled and boiled aqueous hop essential oil solutions.

	Average peak area/hop oil concentration[1]	CV (%)[2]	Average relative area (%)	CV (%)	Average recovery upon boiling[3]	CV (%)
Unboiled samples						
Monoterpene hydrocarbons	19942599	3	24	2	-	-
Floral compounds	2572412	6	3	3	-	-
Sesquiterpene hydrocarbons	60611769	3	72	1	-	-
Spicy compounds	1246299	4	1	3	-	-
Boiled samples						
Monoterpene hydrocarbons	9406330	4	22	4	47	4
Floral compounds	3342765	2	8	2	130	5
Sesquiterpene hydrocarbons	26245365	3	63	2	43	4
Spicy compounds	2874676	4	7	5	231	5

[1] Hop essential oil concentration: 75 mg/L, cv. Saaz. [2] CV (%)= coefficient of variation. [3] Recovery: relative measurement for the amount found in boiled hop essential oil samples compared to unboiled samples, calculated by dividing the average 'peak area (of each chemical compound class) to hop oil concentration ratio' of the boiled samples by the average 'peak area to hop oil concentration ratio' of the unboiled samples.

Exploratory data analysis (EDA) represents a group of multivariate data analysis methods that are capable of visualizing structure in a data block by reducing the dimensionality, which is optimally achieved by removing noise while retaining the meaningful information. EDA methods include both principal components analysis (PCA) and cluster analysis (CA)[179]. We performed PCA on a data matrix constructed with the 'peak area to total hop essential oil concentration ratio' of each compound class for the 8 samples. The data matrix was pre-processed by autoscaling and 2 PCs were selected, explaining 99.8 % of the variance. The scores in the biplot (see **Figure 2-2**) demonstrate a clear clustering of unboiled versus boiled samples. PC 1 explains the variance between the 2 clusters (98.4 % of the variance), whereas PC 2 explains the variance within a cluster (1.4 % of the variance). On the basis of the loadings, it could further be concluded that unboiled samples are characterised by high levels of terpene hydrocarbons, whereas the loadings of the spicy and floral compound classes were found in close proximity of the boiled samples.

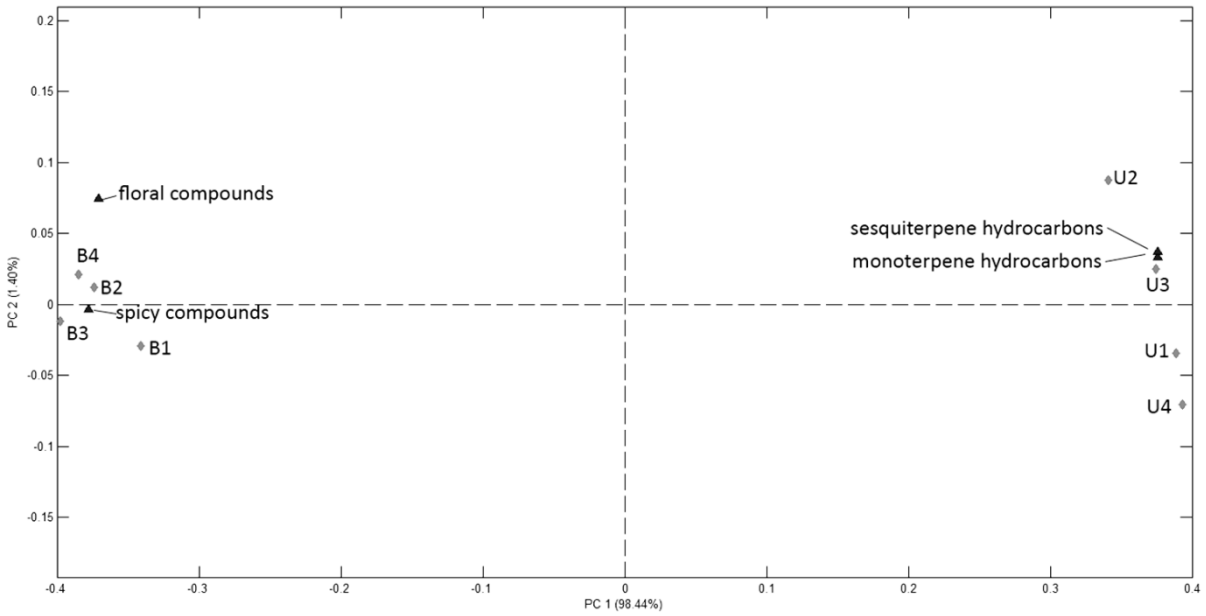


Figure 2-2. Biplot of principal component analysis on unboiled (U1-U4) and boiled (B1-B4) hop essential oil samples cv. Saaz on the basis of area-concentration ratio (see Table 2-1) of different compound classes. Preprocessing=autoscaling, 2 PCs account for 99.8 % of the variance. Samples are represented as scores, and compound classes as loadings.

CA, also called unsupervised pattern recognition (PARC), is another EDA technique, which reduces the dimensionality by producing a representation of the closeness or similarity between objects/samples, using distances in the multidimensional factor space. Hierarchical clustering is a variation of CA which represents the results in a dendrogram to indicate the degree of similarity between samples. Hierarchical clustering can be further subdivided into agglomerative and divisive methods where the agglomerative methods start from each sample as a separate group and then merge the closest groups to clusters, whereas the divisive methods do the opposite and start with all the samples in one group and then split the group into clusters[179,186,187,190].

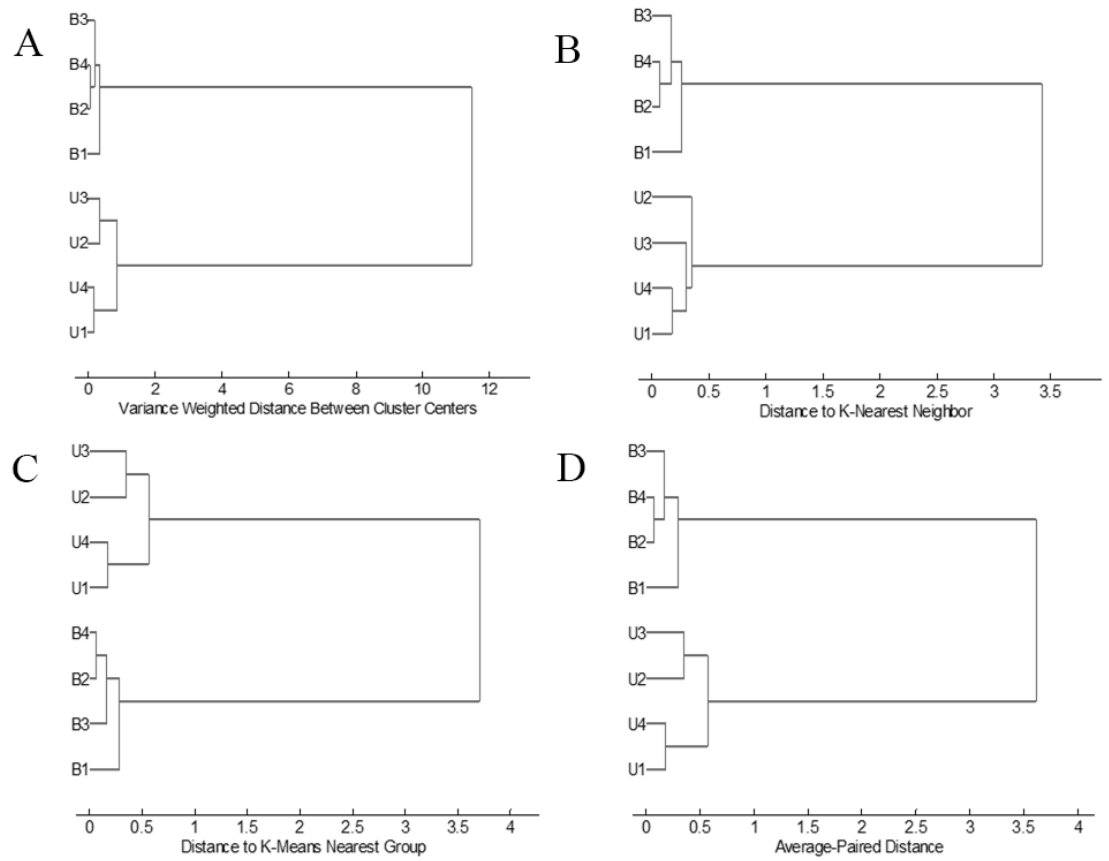


Figure 2-3. Dendrograms obtained after hierarchical agglomerative clustering of boiled (B1-B4) and unboiled (U1-U4) samples on basis of area-concentration ratio (see Table 2-1) of different compound classes. Preprocessing= autoscaling, noise-filtering by selecting 2 principal components (accounting for 99.8 % of the variance). A= Ward's method, B= K-nearest neighbour, C= agglomerative K-means, D= average paired distances.

Our data matrix was subjected to different hierarchical agglomerative clustering methods, including Ward's method, unsupervised K-nearest neighbour, agglomerative K-means, and average paired distances. Euclidean distances in the PCA-transformed space (2 PCs, explaining 99.8 % of the variance) were used. Ward's method, K–nearest neighbour, agglomerative K-means and average paired distance method, yielded highly similar results, although the algorithm used for clustering is different and each method performs optimal for a particular type of clusters (*e.g.* 'round' clusters versus 'chain-type' clusters). The four resulting dendrograms (**Figure 2-3**) depict which samples were joined into which cluster as a function of the distance between samples. If there is a long gap between two distances at which samples are joined into clusters, this is a sign of a clear group structure[190]. It can be seen in all dendrograms that there is an obvious discrimination between unboiled and boiled samples, although Ward's method showed to give the best clustering performance.

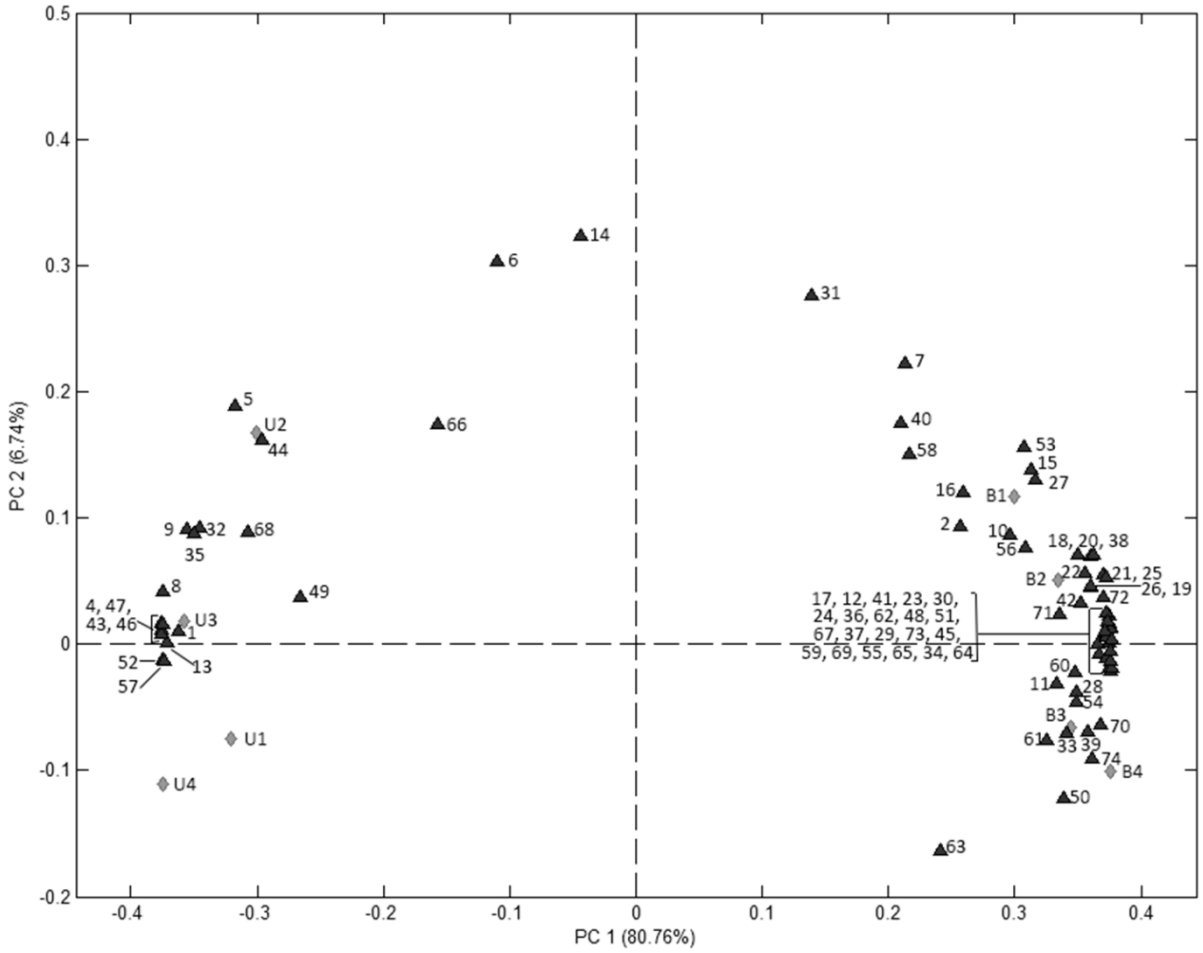


Figure 2-4. Biplot of principal component analysis on unboiled (U1-U4) and boiled (B1-B4) hop essential oil samples cv. Saaz on basis of normalised peak areas (*i.e.* peak areas obtained via HS-SPME-GC-MS analysis, divided by the added hop oil concentration) for individual volatiles.

Preprocessing=autoscaling, 2 PCs account for 87.50% of the variance. Samples are represented as scores, and compound classes as loadings. (Tentatively) identified compounds in unboiled (U) and boiled (B) samples: (1) α-pinene[R], (2) camphene[R], (3) β-pinene[R], (4) β-myrcene[R], (5) 3-methylbutyl isobutanoate[R], (6) methyl heptanoate[R], (7) p-cymene[R], (8) limonene[R], (9) *cis*-β-ocimene[R], (10) γ-terpinene[R], (12) terpinolene[R], (13) nonanal[R], linalool[R], (14) perillene, (16) methyl octanoate[R], (17) linalyl ethyl ether, (18) 2-decanone[R], (19) dodecane, (20) methyl 3-nonenoate[R], (22) methyl nonanoate, (25) α-terpinyl ethyl ether, (26) 5-undecen-2-one, (27) methyl 4,6-dimethyloctanoate, (29) 2-undecanone[R], (30) methyl *trans*-4-decenoate, (32) α-cubebene, (35) (1R,8R,9S)-5,8-cyclocaryophyll-4-ene, (36) α-ylangene, (37) α-copaene, (42) *cis*-α-bergamotene, (43) β-caryophyllene[R], (44) β-copaene, (45) *trans*-α-bergamotene, (46) β-farnesene[R], (47) α-humulene[R], (48) γ-muurolene, (49) α-amorphene, (51) β-selinene, (52) γ-amorphene, (53) α-muurolene, α-selinene, (54) β-bisobolene, (55) γ-cadinene, *trans*-calamenene, (56) δ-cadinene, (57) zonarene, (58) *trans*-cadina-1,4-diene, (59) α-calacorene, α-cadinene, (61) selina-3,7(11)-diene, (63) (E)-dendrolasin, B germacrene, (64) caryolan-1-ol, humuladienone, (65) 6(5→4)-abeo-caryophyll-8(13)-en-5-al, (66) caryophyllene oxide[R, cep], (67) humulene epoxide I[hep], humulol, (68) humulene epoxide II[hep], (70) humulene epoxide III[hep], humulenol II[haa], (71) τ-cadinol, (74) pentadecanone. [R]= use of reference compound for identification. cep/hep= caryophyllene/humulene epoxide, found in reference mixture (**section 2.2.1.2**). haa= humulene derived allylic alcohol, found in reference mixture (**section 2.2.1.2**).

Next, multivariate analysis was performed on a dataset, containing the 4 boiled and 4 unboiled samples as objects and the 'peak area - total hop essential oil concentration' ratio of individual volatiles as variables. In the first instance, the dataset was explored by performing PCA. Two PCs accounted for 87.5 % of the variance and the biplot (see **Figure**

2-4) revealed that unboiled samples are characterised by higher levels of α-humulene, β-caryophyllene, β-myrcene and β-farnesene, whereas boiled samples are characterised by higher levels of OSs. Surprisingly, caryophyllene oxide and humulene epoxide II (see **Figure 2-4**, n° 66 and 68) are oxygenated sesquiterpenoids (OSs) but showed slightly different behaviour compared to the other OSs, since their loadings were located further away from the boiled samples' scores on the biplot. Apparently, these compounds are formed to a lesser extent or they could possibly also be further converted during boiling by hydrolysis and/or oxidation reactions. The sensitivity of humulene epoxide II to hydrolysis and rearrangement reactions has already extensively been demonstrated[10,17,138,173]. Also caryophyllene oxide can easily be isomerised/hydrolysed into a series of products[85]. Besides OSs, the boiled samples are related to the floral fraction since the loadings of floral compounds were in general located closely to the boiled samples' scores. Basically, it can be concluded that boiling of hop oil leads to significant changes in the hop oil composition, and that lower amounts of terpene hydrocarbons and higher amounts of oxygenated terpenoids are typical for boiled hop essential oil, compared to unboiled hop oil. The higher levels of oxygenated terpenoids in boiled samples are most likely explicable by oxidation of terpenes during the boiling process.

Next, it was verified whether unsupervised hierarchical agglomerative cluster analysis with Ward's method on the same dataset would deliver the same clusters (unboiled vs. boiled hop essential oil) as was found on the dataset consisting of the compound classes instead of the individual compounds. Selection of 2 principal components accounted for 87.5 % of the variance and delivered an even more pronounced discrimination between unboiled and boiled hop oil samples (**Figure 2-5**), compared to the use of the compound classes (**Figure 2-3 A**).

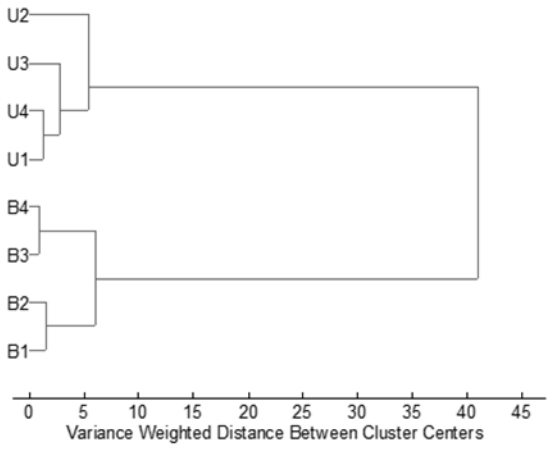


Figure 2-5. Dendrogram obtained after hierarchical agglomerative clustering of boiled (B1-B4) and unboiled (U1-U4) samples using Ward's method on basis of area-concentration ratio of individual compounds.
Preprocessing=autoscaling, noise-filtering by selecting 2 principal components (accounting for 87.5 % of the variance).

Finally, we aimed at determining individual compounds most important for the discrimination between unboiled and boiled hop essential oil solutions. The number of variables is very high compared to the number of samples, which is a common practical problem. Most likely, there is the presence of several 'noisy' non-informative and/or correlated variables[191]. Ideally, one could find a reduced subset of the original variables without losing information. The compounds in this reduced subset would be the compounds that are most important for discrimination between clusters. There are some methods available for variable selection, such as the jack-knife method, genetic algorithms and interval PLS (forward and backward)[190]. However, these methods are used to find the most informative subset of variables to construct a calibration model (Partial Least Squares Regression (PLS), Principal Component Regression (PCR), Multivariate Linear Regression (MLR)). Also the so-called VIP-score method (variable importance in projection) estimates the importance of each variable in the projection used in a PLS model. Variables with a VIP score equal or greater than 1 are considered important in the model. However, PLS requires both an X matrix with independent variables and a Y matrix with dependent variables. Because in our case there is only one data matrix (X matrix) and, since we are dealing with highly clustered data, we propose the use of a supervised pattern recognition (PARC) technique. Techniques frequently used for supervised PARC include nearest neighbour analysis, discriminant analysis (DA), and soft independent modeling of class analogies (SIMCA)[179]. SIMCA is a very useful classification tool but computes PCA sub-models to capture variation within each class without identifying directions in the data space that discriminate classes directly. On the other hand, Partial Least Squares Discriminant Analysis (PLS-DA) creates a Y-matrix with a variable for each class (1=sample belongs to the class, 0= sample does not belong to the class)[192] and offers the possibility to review the loadings (variables) by plotting the VIP scores or selectivity ratio for the Y-variables as a function of the original variables. PLS-DA was performed on the data matrix and the unboiled and boiled samples were separated into 2 different classes. Two latent variables were selected, explaining 85.7 % of the variance within the X matrix and almost 100 % of the variance within the Y matrix. The root mean square error of calibration (RMSEC) and cross validation (RMSECV), R^2 (coefficient of determination) of calibration and R^2 of cross validation were respectively 0.0075, 0.0541, 0.9998 and 0.9894. The loadings were reviewed by plotting the selectivity for the predicted class (Y) as a function of the variables. This plot is depicted in **Figure 2-6** and shows the importance of each variable (numbering in accordance with **Figure 2-4**) for assignment of an unknown sample to a particular class (boiled or unboiled). The variables associated with high peaks in this plot are candidates for being the most discriminating compounds, although conclusive statements regarding which compounds are the most important are not straightforward to make (due to the high number of variables

compared to the analysed samples). It can also be seen that the most discriminating variables are not necessarily the compounds with the largest peak area, since *e.g.* peak n°67 (co-elution of humulene epoxide I and humulol) shows the highest selectivity but has a much smaller 'peak area to concentration ratio' than for example α-humulene (n°47) (**Figure 2-6**).

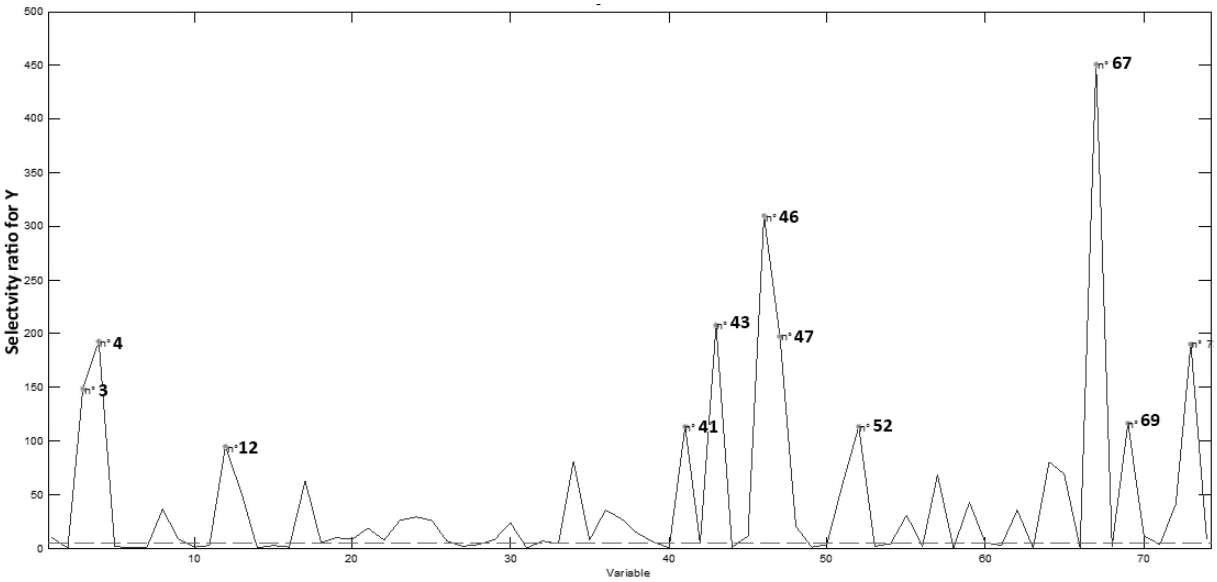


Figure 2-6. Selectivity for predicted class plotted as a function ot the original variables, obtained after performing PLS-DA (objects=unboiled and boiled hop oil samples, variables= individual compounds). Preprocessing= autoscaling, selection of 2 LV's. Compound numbering in accordance with **Figure 2-4**.

In an attempt to control the PLS-DA results, Ward's method was performed on a subset of the original data matrix, containing only the 11 variables with the highest selectivity for the predicted class. Two PCs were chosen and because the number of variables was significantly reduced, noise was also reduced and the explained variance increased to 99.7 %, compared to 87.5 % when using the complete data set. The clustering between unboiled and boiled samples was still very clear, pointing to limited information loss, although the variance weighted distance between the cluster centres decreased from approximately 40 (**Figure 2-5**) to 17 when using the subset of variables. In conclusion, PLS-DA proved to be a useful multivariate analysis technique for selection of variables that are most important for discrimination between unboiled and boiled hop essential oil samples.

2.3.2 Analytical investigation of quantitative and qualitative differences between unboiled and boiled aqueous hop essential oil solutions (cv. Saaz)

To further investigate the observed analytical discrimination between unboiled and boiled hop essential oil over a broad concentration range, increasing concentrations of unboiled and boiled hop essential oil (see **section 2.2.5.1**) were analysed by liquid injection of the SPE-eluate into the GC-MS. Whereas HS-SPME is an extraction technique based on a partition

coefficient (liquid-gaseous phase and gaseous phase- absorption on the fibre) which might be influenced by the presence of other compounds, liquid injection is not based on an equilibrium and may prove useful to verify the increase in the spicy fraction upon boiling of hop essential oil, observed upon HS-SPME sampling. In **Figure 2-7**, the results obtained on the monoterpene hydrocarbons (**A**), floral compounds (**B**), SHCs (**C**), and spicy fraction (**D**), are depicted separately. It can be noticed in graph **A** that the peak area ratio of the monoterpene hydrocarbon fraction is more or less equal for unboiled and boiled samples at the lowest concentrations. However, as the concentration of boiled hop essential oil is increased, the graphs indicate that the level of monoterpene hydrocarbons decreases during the boiling process. The SHCs (**C**) shows the same behaviour. The graph representing the floral hop essential oil compounds (**B**) indicates that the total level of floral compounds does not significantly increases during boiling. Nevertheless, formation of monoterpene-derived oxidation products (*e.g.* oxidation of β-myrcene into linalool, nerol, geraniol and citral[136]) and conversion of particular compounds such as oxygenated monoterpenoids into other oxidised derivatives[137] might still occur.

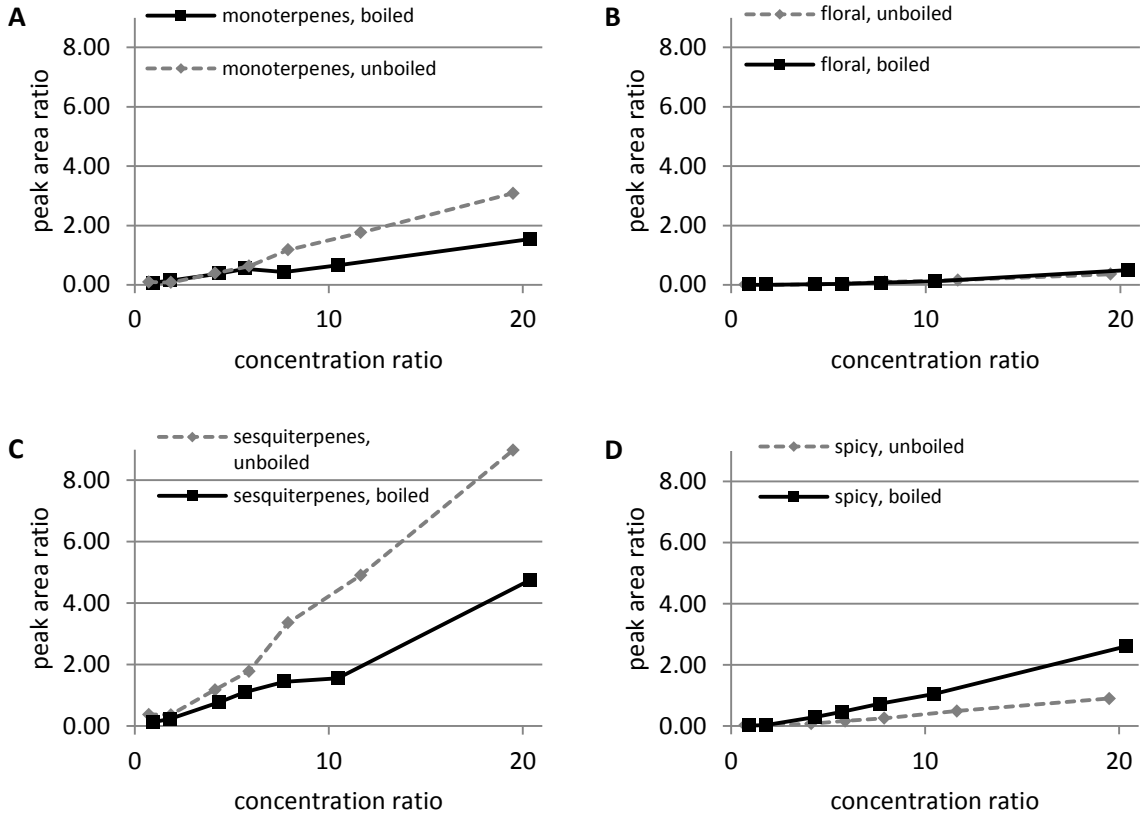


Figure 2-7. Peak area ratio as a function of concentration ratio for monoterpene hydrocarbons (A), floral compounds (B), sesquiterpene hydrocarbons (C) and spicy compounds (D) of unboiled and boiled samples with increasing hop essential oil concentration (0.8, 1.7, 3.3, 4.9, 6.3, 8.9 and 14.3 g/L hop essential oil boiled in aqueous matrix, transferred via SPE to ethanolic solution and analysed via liquid injection GC-MS).
Peak area ratio: peak area of compound class divided by peak area internal standard. Concentration ratio: original hop essential oil concentration divided by internal standard concentration.

Interestingly, graph **D** clearly demonstrates a significant increase in the level of spicy compounds upon boiling, within the complete investigated concentration range. This confirms the assumption that SHCs are oxidised into OSs during boiling. Formation of OSs as a result of reflux boiling of α-humulene and β-caryophyllene was already demonstrated by Peacock and Deinzer[17]. However, we have experimentally shown for the first time an increase in the level of spicy compounds (incl. OSs) as a result of lab scale boiling of total hop essential oil. Furthermore, since the graphs for boiled and unboiled samples diverge (see **Figure 2-7 A**, **C**, **D**) as the added hop oil concentration is increased, for both terpenes and spicy compounds, it can be concluded that the volatile profile from boiled hop essential oil differentiates more from unboiled samples as a function of increasing hop oil concentration.

Recoveries of marker compounds (selected to represent the behaviour of other compounds belonging to the same chemical compound class) in the volatile profile of u2 and b2 (prepared as described in **section 2.2.5.2**) were calculated (see **Table 2-2**). The total level of monoterpene hydrocarbons decreased upon boiling, which is also reflected by the recovery of β-pinene, β-myrcene, limonene and *cis*-β-ocimene. The decrease in the total level of SHCs was more pronounced, which can be mainly attributed to the low recoveries of the 2 major SHCs, β-caryophyllene and α-humulene (recoveries of resp. 55 % and 56 %). Most of the SHCs (represented by α-copaene) depicted slightly higher recoveries, however still indicating a decrease in their level upon boiling. The recoveries of clovene (135 %), a β-caryophyllene rearrangement product[193], and cadalene (209 %), a rearrangement product of α-gurjunene[194], suggest that some SHCs may be transformed into other SHCs via rearrangement reactions. Amongst the floral compounds, one oxygenated monoterpenoid (perillene) with a recovery significantly higher than 100 % after boiling was detected. In contrast to the other hop oil fractions, the spicy fraction exhibits a clear increase in the total level upon boiling, mainly due to high recoveries of particular oxygenated β-caryophyllene and α-humulene derivatives (see **Table 2-2**). The recoveries of humulene epoxide III, humulol and caryophylla-4(12),8(13)-diene-5-ol (occurrence of co-eluting peaks) were estimated by filtering the chromatograms (selected ion monitoring) on specific mass fragments (resp. m/z 81; m/z 82 and 83; m/z 136), resulting in recoveries of 293 %, 167 % and 278 % respectively. Various α-humulene and β-caryophyllene oxidation products have already been associated to the herbal/spicy character of hoppy aroma since decades[10,17–19,27]. Increases in levels of these compounds might also occur during kettle boiling in real brewing practice and these increases may contribute to the development of 'noble' kettle hop aroma, which is typically imparted by a long boil using European/noble hop varieties. Cadinols (*e.g.* τ-cadinol), which were suggested to be derived from the hop plant biosynthesis[18] rather than from chemical

oxidation, did not show an increase in their level upon boiling and the same could be observed for OSs with a similar structure (*e.g.* 1-epi-cubenol).

Table 2-2. Tentative identification of marker compounds, detected in sample u2 and b2 (1.67 and 1.68 g/L hop essential oil resp.) after SPE-transfer and HS-SPME-GC-MS analysis*.

Compound	RI	R	a	b	LOD (µg/L)	recovery (%) upon boiling	CV %	Identification
β-Pinene	968	0.9936	0.0004	-	2.99	90	5	KI, MS, RC
β-Myrcene	981	0.9929	0.0006	0.0001	0.55	76	14	KI, MS, RC
Limonene	1023	0.9953	0.0006	-	1.59	73	4	KI, MS, RC
cis-β-Ocimene	1039					46	11	KI, MS, RC
Perillene	1089					254	18	KI, MS
2-Undecanone	1275	0.9983	0.0015	-	1.06	73	6	KI, MS, RC
Methyl 4-decenoate	1291					75	11	KI, MS
Methyl geranate	1304	0.9971	0.0006	-	3.53	82	4	KI, MS, RC
Clovene	1352					135	14	KI, MS
α-Copaene	1371					87	11	KI, MS
β-Caryophyllene	1412	0.9987	0.0038	-	0.16	55	9	KI, MS, RC
α-Humulene	1447	0.9989	0.0045	-	0.12	56	6	KI, MS, RC
Humuladienone	1550					313	6	KI, MS
Caryophyllene oxide	1555	0.9994	0.0010	-	2.65	181	7	KI, MS, RC, cep
Humulene epoxide I	1574					264	23	KI, MS, hep
Humulene epoxide II	1584					141	12	KI, MS, hep
1-*epi*-Cubenol	1595					51	7	KI, MS
τ-Cadinol	1622					72	20	KI, MS
Caryophylla-3,8(13)-diene-5β-ol	1644					131	8	KI, MS, caa
Cadalene	1645					209	8	KI, MS
Humulene allylic alcohol	1652					293	16	KI, MS, haa
Total monoterpene hydrocarbons						76	12	
Total floral compounds						88	14	
Total sesquiterpene hydrocarbons						57	5	
Total spicy compounds						147	13	
Total hop essential oil						65	4	

*If the reference compound is available, recoveries are calculated on basis of the levels in u2 and b2, obtained via the calibration curve. R= correlation coefficient of calibration line, a= slope, b= intercept, LOD= limit of detection (=concentration at which signal to noise ratio is 3). CV%= coefficient of variation. Identification on basis of reference compounds (RC), retention index (RI) and mass spectrum (MS). cep/hep= caryophyllene/humulene epoxide, found in reference mixture (**section 2.2.1.2**). caa/haa= caryophyllene/humulene derived allylic alcohol, found in reference mixture (**section 2.2.1.2**).

Analytical characterisation of the volatile profiles of the 10, 100, 500 and 1000 mg/L boiled and 10 mg/L unboiled hop essential oil solutions (see **section 2.2.5.2**) demonstrated qualitative differences. Some compounds, which are listed in **Table 2-3**, were only detected in the boiled samples and not in the unboiled sample, although all samples were analysed at an identical final concentration of 10 mg/L. These findings prove that these compounds are hop essential oil-derived compounds, formed *de novo* upon boiling. Interestingly, the number of new peaks in the boiled samples increases as the initial added hop oil concentration was increased. These results confirm that boiling of higher concentrations of hop essential oil leads to more pronounced differences in the hop-derived volatile profile.

The presence of particular SHCs (**Table 2-3**, **n° 7**, **8**, **9**, **10**, **12**) in boiled hop essential oil samples suggest that SHCs can be converted into other SHCs, whereas some

monoterpenoids (**n° 1**, **6**, **3**, **5**) in the boiled samples indicate that the floral fraction also undergoes qualitative changes upon boiling. Karahana ether (**n° 2**), which was already demonstrated to increase during storage[17,82], is also only found after boiling.

In this work, we also detected a series of newly formed OSs in the boiled samples. Humulol was detected in all boiled samples but not in the unboiled samples, probably due to levels below the detection limit. This compound is present at low levels in hops but is more prominent in beer[17,28,88], pointing to an increase in its level during the brewing process and during ageing of hops[82]. Compound **n° 14** (1,5,8,8,-tetramethyl-12-oxa-5-tricyclo-[7.2.1.$0^{6,9}$]-dodecene) and **n° 20** (4,8,11,11-tetramethyl-8-tricyclo-[7.2.0.$0^{2,5}$]-undecen-4-ol) are humulene epoxide II/III hydrolysates[138] and both have also been detected in beer[84]. The caryophyllene derivatives 4S-dihydrocaryophyllene-5-one (**n° 16**) and 6(5→4)-abeo-8,12-cyclo-caryophyllan-5-al (**n° 17**) were detected in hop oil by Yang and coworkers[85]. Detection of OSs characterised by an increase upon lab scale boiling in lager beer would not prove oxidation during wort boiling since these compounds are already present in unboiled hop oil. However, detection of OSs that are characteristic for boiled hop oil, thus formed *de novo* upon boiling, in commercial kettle hopped lager beers would unambiguously prove that these compounds are also formed during kettle boiling of hopped wort and, consequently, that our current lab scale experiments are indeed relevant to brewing practice (see later **Chapter 3**).

Table 2-3. Determination of newly (*de novo*) formed compounds upon boiling of hop essential oil (cv. Saaz)*.

Compounds not detected in unboiled hop oil	RI calc	10 mg/L	100	500	1000	n°	Identification
Unknown monoterpenoid (m/z 67, 71, 79, 81, 93, 107, 122)	1031			x	x	1	
Karahana ether	1044			x	x	2	KI, MS
Linalyl ethyl ether	1181			x	x	3	KI, MS
Unknown (m/z 43, 81, 99, 127)	1226			x	x	4	
α-Terpinyl ethyl ether	1247		x	x	x	5	KI, MS
Unknown monoterpenoid (m/z 69, 93, 121, 136)	1253			x	x	6	
Unknown sesquiterpene hydrocarbon (m/z 91, 105, 119, 147, 175, 190, 204)	1310		x	x	x	7	
Unknown sesquiterpene hydrocarbon (m/z 91, 105, 119, 147, 175, 190, 204)	1322	x	x	x	x	8	
1R,8R,9S-5,8-Cyclocaryophyll-4-ene	1359	x	x	x	x	9	KI, MS
5,8-Cyclo-(1R,5S,8R,9S)-caryophyll-4(12)-ene	1364	x	x	x	x	10	KI, MS
Unknown oxygenated sesquiterpenoid (m/z 79, 93, 107, 121, 135, 145, 163, 205, 220)	1397	x	x	x	x	11	
cis-α-Bergamotene	1408		x	x	x	12	KI, MS
Unknown oxygenated sesquiterpenoid (m/z 137, 205, 220)	1435		x	x	x	13	hhp
1,5,8,8,-Tetramethyl-12-oxa-5-tricyclo[7.2.1.$0^{6,9}$]dodecene	1467		x	x	x	14	KI, MS
Unknown (m/z 177, 220)	1520		x	x	x	15	
4-S-Dihydrocaryophylllene-5-one	1530	x	x	x	x	16	KI, MS
6(5→4)-Abeo-8,12-cyclo-caryophyllan-5-al	1530		x	x	x	17	KI, MS
Unknown oxygenated sesquiterpenoid (m/z 93, 205, 220)	1540	x	x	x	x	18	
Humulol	1579	x	x	x	x	19	KI, MS
4,8,11,11-Tetramethyl-8-tricyclo-[7.2.0.$0^{2,5}$]undecen-4-ol	1583		x	x	x	20	KI, MS
Humulene allylic alcohol (m/z 93, 109, 177, 205, 220)	1593	x	x	x	x	21	haa
Unknown (m/z 139)	1602			x	x	22	hhp
Unknown oxygenated sesquiterpenoid (m/z 93, 107, 133, 159, 187, 202, 248)	1613		x	x	x	23	
Number of compounds characteristic for boiled hop oil (not detected in unboiled sample		**8**	**17**	**23**	**23**		

*Boiled hop essential oil solutions of 100, 500 and 1000 mg/L were diluted to the same final concentration of 10 mg/L before HS-SPME-GC-MS analysis. X= detected in particular sample. RI = calculated retention index. hhp= humulene epoxide hydrolysis product, found in reference mixture (section 2.1.2.2). haa= humulene derived allylic alcohol, found in reference mixture (**section 2.2.1.2**).

2.3.3 Sensorial evaluation of boiled hop essential oil in non-aromatised iso-α-acid bittered lager beer

Preliminary sensory evaluations showed that the 1000 mg/L boiled hop essential oil dilution expressed interesting flavour characteristics, associated to 'hoppy' notes rather than to the aroma of unprocessed hop oil. Therefore, reference beers (without additon of hop oil) were sensorially compared to beers with addition of boiled hop essential oil by our trained taste panel in two independent descriptive tests. The results are displayed via spider plots in **Figure 2-8**. In both sessions, the non-aromatised reference beer was described as 'malty-wort', 'fruity' and somewhat 'floral'. Interestingly, the beers with the addition of boiled hop essential oil cv. Saaz (addition rate: 1 mg/L) were described as 'citrusy', 'spicy' and 'hoppy', although the spicy effect was apparently more perceived during the first session. The beers spiked with boiled hop oil did not express the hay/straw notes, characteristic for unboiled hop oil but, in contrast, were clearly associated with 'hoppy' aroma.

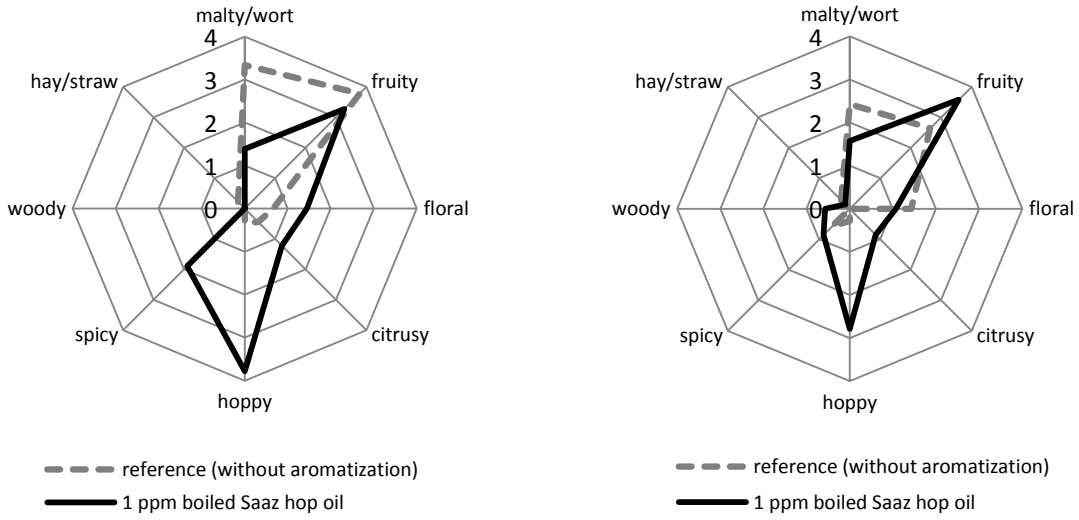


Figure 2-8. Spider plots, showing the average (n=12) of individual scores for selected descriptors, used for sensorial evaluation of the boiled hop essential oil fraction (cv. Saaz) in non-aromatised iso-α-acid bittered pilsner beer. Each spider plot represents the results of a separate sensorial session.

2.4 Conclusions

So far, boiling experiments with total hop essential oil as carried out in this PhD, have not been reported. This methodology, in combination with HS-SPME-GC-MS analysis and the use of multivariate statistics, has allowed us to demonstrate analytical differences between the volatile profile of unboiled and boiled hop essential oil. PCA delivered biplots on which clear clustering of the unboiled and boiled samples could be observed. Subsequently, CA was employed to classify these samples in groups. When comparing unboiled and lab scale boiled total hop essential oil (cv. Saaz), an increase in the level of 'spicy' compounds in the boiled samples compared to the unboiled samples could be observed for the first time and this observation appeared valid over a broad concentration range. In particular, boiled hop essential oil was characterised by higher levels of oxygenated α-humulene and β-caryophyllene derivatives, suggesting oxidation of SHCs as a consequence of boiling. Moreover, a series of compounds, newly formed upon boiling and thus characteristic for boiled hop essential oil, were (tentatively) identified. Many of these components have not been determined before. These compounds might be an important tool to prove oxidation of SHCs during the wort boiling process of real brewing practice. Furthermore, the flavour profile of non-aromatised iso-α-acid lager beers spiked with boiled hop essential oil clearly shifted towards descriptors such as 'spicy' and 'hoppy'. Changes in the volatile profile of hop essential oil as a consequence of boiling may therefore play a huge role in the difference between the aroma of raw hops and the kettle hoppy aroma present in beer. Therefore, the newly formed compounds and the terpene oxidation products that increase in their level upon boiling, are candidates for key character impact compounds of 'hoppy' aroma in beer.

Chapter 3

SPE FRACTIONATION AND CHARACTERISATION OF HOP ESSENTIAL OILS (UNBOILED VS. BOILED) AND HOP OIL-DERIVED VOLATILES IN COMMERCIAL KETTLE HOPPED LAGER BEERS

A part of chapter 3 corresponds to:

Praet,T.; Van Opstaele, F.; Baert, J.; Aerts, G. and De Cooman, L.

Comprehensive characterisation of the hop-derived sesquiterpenoid fingerprint of American kettle hopped lager beers.

BrewingScience, 67:183-194, **2014**

Chemical-analytical profiling of SPE-derived fractions of unboiled and lab scale boiled hop essential oils (cv. Saaz) in aqueous solutions. Sensory evaluation of fractions by GC-O analyses and through descriptive tests by a taste panel. SPE fractionation of hop oil-derived compounds of commercial kettle hopped lagers, identification of compounds in the spicy fraction and determination of flavour-active intervals via GC-O.

Contributions

Tatiana Praet performed the experiments. The manuscript was written by Tatiana Praet and revised and adapted after critical input by Dr. Filip Van Opstaele and Prof. Luc De Cooman.

3 SPE FRACTIONATION AND CHARACTERISATION OF HOP ESSENTIAL OILS (UNBOILED VS. BOILED) AND HOP OIL-DERIVED VOLATILES IN COMMERCIAL KETTLE HOPPED LAGER BEERS

3.1 Introduction

Years of extensive scientific research piled up a significant amount of data supporting the impact of oxygenated sesquiterpenoids (OSs) on hoppy aroma of beer. However, difficulties remain to actually pinpoint the compounds responsible. The reasons for that are quite fundamental and range from the potential occurrence of synergetic effects[7,8,32,113,195], lack of reference compounds and high quality mass spectra, to high degrees of co-elution hampering allocation of a perceived aroma to a particular compound when performing GC-O analyses[114,124]. Another obstacle that should not be underestimated is the relatively low level of OSs that survive the brewing process and is found in the final beer (typically in the range of 10 to 100 ppb). Basically, determining the impact of OSs on hoppy aroma is challenging since the detection limits of chromatographic systems do not always permit a clear detection of some particular compounds in beer. This was certainly a bottle-neck in the early years of hop essential oil research and, indeed, various researchers reported on extraction of large volumes of beer and concentration methods prior to GC-MS analysis for detection and identification of hop oil volatiles in beer[10,16–18,26,83,85,139,173,183,196]. Nowadays, advanced techniques such as headspace SPME are employed to extract and concentrate volatiles from hops, hop essences, and beer samples[6,146,172,197,198]. SPME has proven to be a rapid and solvent free extraction method. Nevertheless, at present, the number of papers in which an attempt is made to characterise the detailed spectrum of OSs, is very limited[19,124].

Since boiled hop oil showed interesting flavour characteristics regarding 'kettle hop' aroma when added to beer (**Chapter 2**), in this chapter we aim at detailed analytical and sensory characterisation of both unboiled and lab scale boiled hop essential oil (cv. Saaz). To (partially) overcome the problem of co-elution when performing monodimensional GC and aiming at a more focused search for compounds that might impart 'kettle hop' aroma, we developed a methodology based on solid phase extraction (SPE) for fractionation of compounds in hop oil extracts. In addition, the same approach is applied for comprehensive analytical characterisation of the sesquiterpenoid fingerprint of three commercial kettle hopped lager beers.

3.2 Experimental

3.2.1 Chemicals

All reference compounds were purchased from Sigma Aldrich (St. Louis, MO, USA) and were analytical grade:

2-decanone (99.5%); 2-heptanol (98%); 2-nonanone (99.5%); 2-tridecanone (97.0%); 2-undecanone (99.0%); 2-dodecanone (97.0%); 3-carene (≥90%); 3-methylbutyl 2-methylpropanoate (≥98%); camphene (95.0%); caryophyllene oxide (≥99.0%); decanal (≥98.0%); dodecanol (≥98.0%); ethyl isovalerate (≥98%); ethyl nonanoate (≥98.0%); geraniol (98%); geranyl acetate (≥97%); limonene (97.0%); linalool (98.5%); methyl 3-nonenoate (99.8%); methyl decanoate (99.5%); methyl geranate (98%); methyl heptanoate (≥99.0%); methyl nonanoate (99.8%); methyl octanoate (99.8%); nonanal (95.0%); ocimene (≥90.0%, mixture of isomers); p-cymene (≥99.0%); terpinolene (≥90.0%); *trans*-nerolidol (98.0%); *trans*-β-farnesene (≥90%); α-copaene (≥90%); α-humulene (≥98.0%); α-pinene (98.0%); β-caryophyllene (98.5%); β-myrcene (≥95.0%); β-pinene (99.0%); γ-terpinene (≥97.0%); terpinen-4-ol (≥95.0%)

Ethanol absolute (≥ 99.8%) was purchased from VWR International (Zaventem, Belgium); MQ-water was obtained from a MQ purification system (Synergy 185, Millipore S.A., Molsheim, France); Sodium chloride was purchased from Merck (for analysis, 1 kg, Darmstadt, Germany).

3.2.2 Plant Material

Saaz hop pellets T90 (crop year 2012) were provided by the Barth-Haas Group (Joh. Barth & Sohn GmbH & Co. KG, Nürnberg, Germany). For storage conditions, see **section 2.2.2**.

3.2.3 Commercial lager beers

Three kettle hopped commercial lager beers (A, B and C) expressing a distinct kettle hoppy aroma, were obtained from the USA and stored at 4°C until analysis. The alcohol percentage (v/v%) of beer A, B and C is 5.20%, 4.90% and 4.90%, respectively, bitterness amounts to 30 IBU, 30 IBU and 32 IBU, respectively, whereas the original gravity is 13°P, 13°P and 12°P, respectively. For beer A, amongst other varieties, the following noble aroma hops were used: Hallertau Mittelfrüh and Vanguard. Beer B was exclusively brewed with the German noble aroma hop varieties Hallertau Mittelfrüh and Tettnang Tettnanger. Beer C was exclusively brewed with the hop variety Northern Brewer.

3.2.4 Solid Phase Extraction (SPE)

3.2.4.1 Fractionation of unboiled and boiled hop essential oil (cv. Saaz)

Hop essential oil was extracted via supercritical fluid extraction as described in **section 2.2.3.2**. The supercritical fluid extract (conc. 5.76 g/L hop oil) was diluted in MQ-water (total volume of 2 mL) in a HS-SPME vial (20 mL, amber glass, Chromacol, Welwyn Garden City, UK) to a final concentration of 1 g/L and the vial was closed with a cap (bimetal magnetic crimp caps containing a silicone/Teflon septum, Interscience, Louvain-la-Neuve, Belgium). Part of the vials remained unboiled, whereas the other part was boiled in the agitator of the CombiPAL autosampler as specified in **section 2.2.4**. Consequently, all vials were opened and further diluted to MQ-water/EtOH solutions (1/1; v/v) by addition of EtOH. Next, the SPE fractionation method as developed by Van Opstaele[15] was followed. Varian Bond Elut C18 cartridges (500 mg, 6 mL, Agilent Technologies, Lake Forest, USA) were placed on a stopcock, inserted in the cover of the SPE manifold. Reduced pressure was obtained by connecting the vacuum port to a water jet pump. The SPE columns were preconditioned with 3 volumes of EtOH, 3 volumes of MQ-water and 3 volumes of MQ-water/EtOH (1/1; v/v), whereupon the content of the vial was pipetted on the column and the eluate collected in a waste container. Next, hop oil compounds adsorbed to the C18 stationary phase were fractionated by gradually increasing the EtOH concentration of the eluent (3 mL) from 50% EtOH/MQ-water (v/v) to 100% EtOH. Each fraction was collected separately, brought into screw-capped brown glass vials (20 mL) and stored in the freezer (-18°C) until further analysis. Fractions from unboiled hop oil that eluted with 50%, 60%, 70%, 80%, 90% and 100% EtOH were encoded U50, U60, U70, U80, U90 and U100, respectively. Similar fractions obtained by SPE fractionation of boiled hop oil were indicated with a 'B'.

3.2.4.2 Isolation and fractionation of hop-derived compounds from lager beer

For detailed analysis of the oxygenated sesquiterpenoid (OS) fraction of 3 commercial kettle hopped lager beers, a Solid Phase Extraction (SPE) based methodology was adapted from the SPE methodology for fractionation of hop oils, developed by Van Opstaele[15]. This method allows for enrichment and fractionation of hop-derived compounds in beer in order to facilitate separation, detection and identification via HS-SPME-GC-MS analysis. Prior to SPE, 200 mL of each beer (A, B and C) was degassed using an ultrasonic bath (Julabo USR 3, Belgolabo, Overijse, Belgium). For each beer, a Bond Elut C18 cartridge (Mega Bond Elut Flash) (1 g, 60 mL, 40 µm, Agilent Technologies, Lake Forest, USA) was employed. The columns were pre-conditioned by eluting respectively 3 volumes of HPLC-grade ethanol, 3 volumes of MQ-water and 3 volumes of an ethanol/MQ-water mixture (5/95; v/v). Degassed beer was pipetted on the column for enrichment of the hop-derived compounds and the

eluate was collected in a waste container. The compounds adsorbed to the column were subsequently eluted with 5 mL HPLC-grade ethanol and the eluate was collected in a vial (10 mL, clear glass, Chromacol, Welwyn Garden City, UK). Next, the eluate of each beer was diluted by addition of 5 mL MQ-water. These dilutions were further fractionated using Bond Elut C18 cartridges (500 mg, 6 mL, Agilent Technologies, Lake Forest, USA). The columns were pre-conditioned with 3 volumes of EtOH, 3 volumes of MQ-water and 3 volumes of EtOH/MQ-water (1/1; v/v), wherupon beer extract was pipetted on the column. The sample eluate was collected in a vial (20 mL, amber glass, Chromacol, Welwyn Garden City, UK) in order to check the absence of hop-derived compounds. Next, adsorbed compounds were fractionated by desorption using an ethanol gradient. Respectively 3 mL of a 50%, 60%, 70%, 80%, 90% and 100% HPLC-grade ethanol/MQ-water mixture (v/v) was pipetted onto the column and each fraction was collected in a separate vial (20 mL, amber glass, Chromacol, Welwyn Garden City, UK). All fractions were stored in the freezer (-18°C) until further analysis.

All fractions were analysed via HS-SPME-GC-MS. For estimation of the level of OSs in the different fractions, the peak areas of the OSs were normalised by taking the internal standard (2-heptanol) into account. In **Figure 3-1**, normalised peak areas are plotted as a function of the analysed fractions. For all the beers, practically no OSs were detected in the sample eluate, implying that the hop-derived compounds were adsorbed onto the column as intended. Fractions eluting with 70 % and 80 % ethanol contained the highest levels of OSs. The OS fingerprint of these particular fractions will be comprehensively characterised in this study.

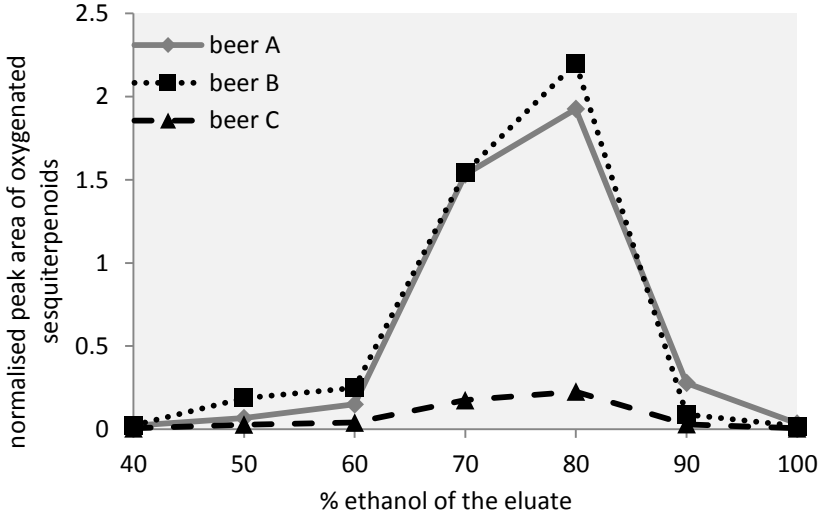


Figure 3-1. Normalised peak area (area sesquiterpenoid fraction / area 2-heptanol (internal standard)) as a measure for the level of oxygenated sesquiterpenoids (OSs), found in the eluate of the sample and fractions with increasing ethanol concentration after Solid Phase Extraction (SPE) of 3 commercial lager beers (A, B, C).

3.2.5 HS-SPME-GC-MS analysis of hop oil-derived and beer-derived SPE fractions

A 10-fold dilution of the hop oil-derived SPE fractions was analysed by addition of 500 µL of the fraction to 4.48 mL of MQ-water and addition of 20 µL internal standard (2-heptanol, stock solution: 12.5 g/L). For quantification of selected marker compounds, stock solutions of reference compounds were made and external calibration curves were drawn up, ranging from 0 to 250 µg/L. Additional 500-fold dilutions of the SPE fractions were made (addition of 10 µL fraction to 20 µL internal standard and 4.97 mL MQ-water), which allows for quantification of the major terpene hydrocarbons β-myrcene, β-caryophyllene, α-humulene and β-farnesene within the linear response range. Fractions (500 µL) derived from SPE fractionation of beers were analysed by adding 4.4 mL MQ-water and 1.5 g sodium chloride in a HS-SPME vial (20 mL, clear glass, Chromacol). Before the vials were closed with bimetal magnetic caps with silicon/Teflon septum (Supelco, Bellefonte, USA), 100 µL 2-heptanol (253 ppm stock solution in ethanol) was pipetted into the vial to a final concentration of 5 ppm to serve as an internal standard. All samples were analysed via HS-SPME-GC-MS using slow oven programming as described in **section 2.2.6**.

3.2.6 Selective quantification of linalool and geraniol in hop oil-derived SPE fractions

Selective quantification of linalool and geraniol via external calibration curves (0-150 µg/L) was performed via a method developed by Van Opstaele[15] using an ion trap mass detector (ITQ 1100, Thermo Fisher Scientific, Austin, TX). Analyses were carried out by operating in the MS/MS mode. The precursor ion at m/z = 121 was isolated and further fragmented by collision induced dissociation (CID; collision energy: 2.0 V; maximum excitation energy q: 0.30, time: 15 ms). The resulting daughter ion at m/z = 105 was selected to quantify the linalool content in the SPE fractions, whereas the daughter ion at m/z 93 was selected for quantification of geraniol.

3.2.7 Determination of flavour-active compounds in SPE fractions via GC-olfactometry

Volatiles of the SPE-derived fractions U60, B60, U70, B70, U80 and B80 and of the fractions eluting with 70% and 80% EtOH upon SPE fractionation of beer B (these fractions contain the highest levels of OSs, see **Figure 3-1**), were extracted via HS-SPME and flavour-active constituents were determined by GC-olfactometry with a Sniffer 9000 system (Brechbüchler Inc., Schlieren, Switzerland), coupled to the GC-MS device. To this end, the experimental conditions were identical to the method used for HS-SPME-GC-MS analysis (see **section**

3.2.5). The effluent from the GC-column was split to the mass spectrometer and the sniffing port (50/50). The temperature of the transfer line (connection GC and sniffing port) was set at 250°C. The effluent was mixed with a stream of humidified air before leaving the sniffing port. Three trained assessors were asked to sniff samples and to indicate flavour-active zones, as well as to record the duration of odour perception via a hand-held control unit with cursor wheel for signal generation. Assessors were trained for detection of OSs by sniffing the OS fraction of hop (oil) and beer samples on a regular basis. Aromagrams were automatically generated via the Xcalibur software. For analysis, 500 µL of a fraction was pipetted into 4,500 µL MQ-water in a HS-SPME vial. Three assessors sniffed the 70% and 80% ethanol fraction derived from beer B, as well the hop oil-derived SPE fractions U60, B60, U70, B70, U80 and B80 (in duplicate). The detection frequency (DF) is calculated as the number of times a particular odour-active zone was detected out of 3 or 6 analyses.

3.2.8 Sensory evaluation of hop oil-derived SPE fractions in non-aromatised iso-α-acid bittered lager beer

Preliminary sensory evaluation of the odour expressed by the fractions B50, B60, B70, B80, B90 and B100 was performed by sniffing the fractions upon 10-fold dilution in MQ-water by a taste panel, which consisted of 4 assessors trained for sensory evaluation of hop oil (fractions). Descriptive sensory evaluation of the fractions U50, B50, U60, B60, U70, B70, U80 and B80 in non-aromatised iso-α-acid bittered lager beer (for beer preparation, see **section 2.2.7**) was performed by an extended taste panel (12 panellists). The hop oil concentration in U50, U60, U70 and U80 was determined semi-quantitatively via GC-FID according to the procedure described Van Opstaele[15]. Analyses were performed in triplicate. Hop oil concentrations were 221 ppm (CV= 1%), 183 ppm (CV= 2%), 71 ppm (CV= 5%) and 24 ppm (CV% 2%) for U80, U70, U60 and U50, respectively. Volumes added to the beer were calculated aiming at a final concentration of 500 ppb of hop oil constituents. For the fractions B50, B60, B70 and B80, added volumes to beer were identical to the corresponding unboiled fractions.

3.3 Results and Discussion

3.3.1 Preliminary sensory evaluation of SPE fractions obtained from boiled hop essential oil (cv. Saaz)

In the previous chapter, the beer with addition of boiled hop essential oil did not express the 'hay/straw' notes typical for unboiled hop oil, but was clearly associated with 'hoppy' aroma. These results indicate that boiling of hop essential oil may have an essential role in the development of 'hoppy' or 'kettle hop' aroma. In order to overcome co-elution of hop-derived volatiles, hop essential oil cv. Saaz was boiled and subsequently fractionated via SPE according to the procedure described in **section 3.2.4.1**. Each SPE-derived fraction (B50, B60, B70, B80, B90 and B100) was diluted in MQ-water (10-fold dilution), encoded (for 'blind' sensory evaluation) and presented to our taste panel (4 trained assessors) for description of the odour. Descriptors used for the odour expressed by the fractions are summarised in **Table 3-1.**

Table 3-1. Descriptors used to define odours of SPE fractions derived from boiled hop essential oil (cv. Saaz).

B50	B60	B70	B80	B90	B100
Madeira	Straw	Hoppy	Green	Weak odour	Weak odour
Solvent	Green	Spicy	Hop aroma	Resinous	Resinous
Paint/glue	Floral	Citrus	Similar to B70	Herbal	Herbal
	Hoppy (weak)		(but less intense)		

Clearly, fraction B50 causes non-desired solvent-like off-flavours which are not detected in unboiled hop oils, indicating that this fraction may contain volatiles that are newly formed upon boiling. Also the fractions B90 and B100 are not interesting in relation to 'hoppy' or 'kettle hop' aroma, since these fractions impart weak resinous and herbal notes, reminiscent of the aroma of hop pellets as such. Fraction B60 was described by 'straw' and 'green' notes but also by 'floral' and 'hoppy', although the latter top note was only weak. Fractions B70 and B80 clearly imparted 'spicy' and 'hoppy' impressions. Fraction B70 was also described as slightly 'citrusy', whereas fraction B80 was characterised by a 'green' odour. Obviously, these SPE-derived fractions with 'hoppy/spicy' odour characteristics allow to narrow our investigation of flavour-active hop oil–derived volatiles that may impart 'kettle hop' aroma.

3.3.2 Precision of SPE-fractionation of unboiled and boiled hop essential oil (cv. Saaz) and composition of resulting fractions

Based on the results obtained in **section 3.3.1**, fractions eluting with 60% EtOH, 70% EtOH and 80% EtOH were selected for further analysis.

The precision of the SPE-fractionation and distribution of compound classes over the different fractions were evaluated by performing the SPE procedure described in **section 3.2.4.1** four times (n=4) on both unboiled and boiled hop essential oil dilutions (1 g/L) in MQ-water. Upon SPE fractionation, samples were analysed by HS-SPME-GC-MS and normalised peak areas (peak areas divided by internal standard peak area for compensation of HS-SPME variations) of different compound classes (monoterpene hydrocarbons, floral fraction, sesquiterpene hydrocarbons (SHCs) and spicy fraction) were calculated (see **Figure 3-2**). Standard deviations (S.D.) of normalised peak areas provide an estimation of the variation on the distribution of compound classes over the different fractions and thus of the SPE procedure. Evidently, for boiled samples, variation due to the boiling step is also included.

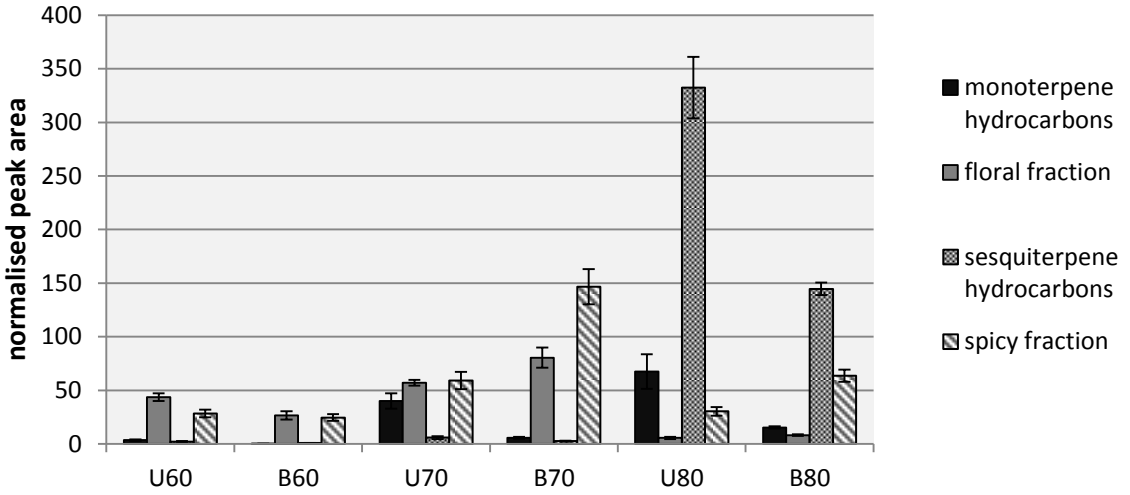


Figure 3-2. Normalised peak areas of chemical compound classes in fractions (60% EtOH, 70% EtOH, 80% EtOH) derived from SPE fractionation of unboiled (U) and boiled (B) hop essential oil cv. Saaz.

Figure 3-3 further shows the composition in terms of compound classes of the fractions B60, B70 and B80.

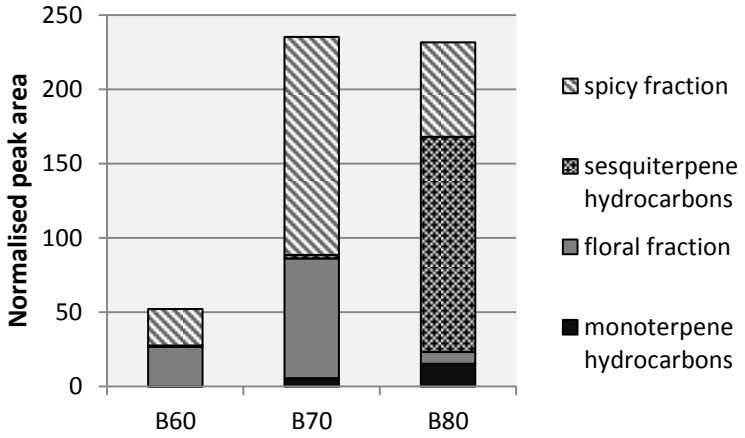


Figure 3-3. Composition (based on normalised peak areas) of fractions B60, B70 and B80.

The composition of B60, B70 and B80 can be related to the descriptors used to charactarise the odour shown by these fractions (see **Table 3-1**). Fraction B70 was defined as 'hoppy' and 'spicy' and seems to be the most interesting fraction with respect to 'kettle hop' aroma. This fraction clearly contains relatively high levels of spicy and floral compounds, further supporting the general view that oxygenated compounds, which largely survive the brewing process and are found in kettle hopped beers, are much more important with regard to 'kettle hop' aroma than the nonpolar terpene hydrocarbons[28,139,157]. Although B80 was found to express a similar yet less intense odour than B70, this fraction was also described in terms of 'green' and 'hop aroma'. Indeed, fraction B80 is characterised by high quantities of monoterpene and in particular SHCs. In general, terpene hydrocarbons express 'resinous' and 'herbal' flavours and compounds such as *e.g.* β-myrcene are key contributors to the aroma of unprocessed hop pellets[4,43,122]. The descriptor 'hoppy' was also assigned to fraction B60 but this flavour characteristic was less intense compared to B70, which can be explainied by the lower level of volatiles in this particular fraction. Fraction B60 especially showed 'floral' odour characteristics, which can be explained by the relatively high share of floral compounds within this fraction and less masking by other volatiles.

3.3.3 Qualitative and quantitative profiling of hop oil-derived SPE fractions via HS-SPME-GC-MS analysis

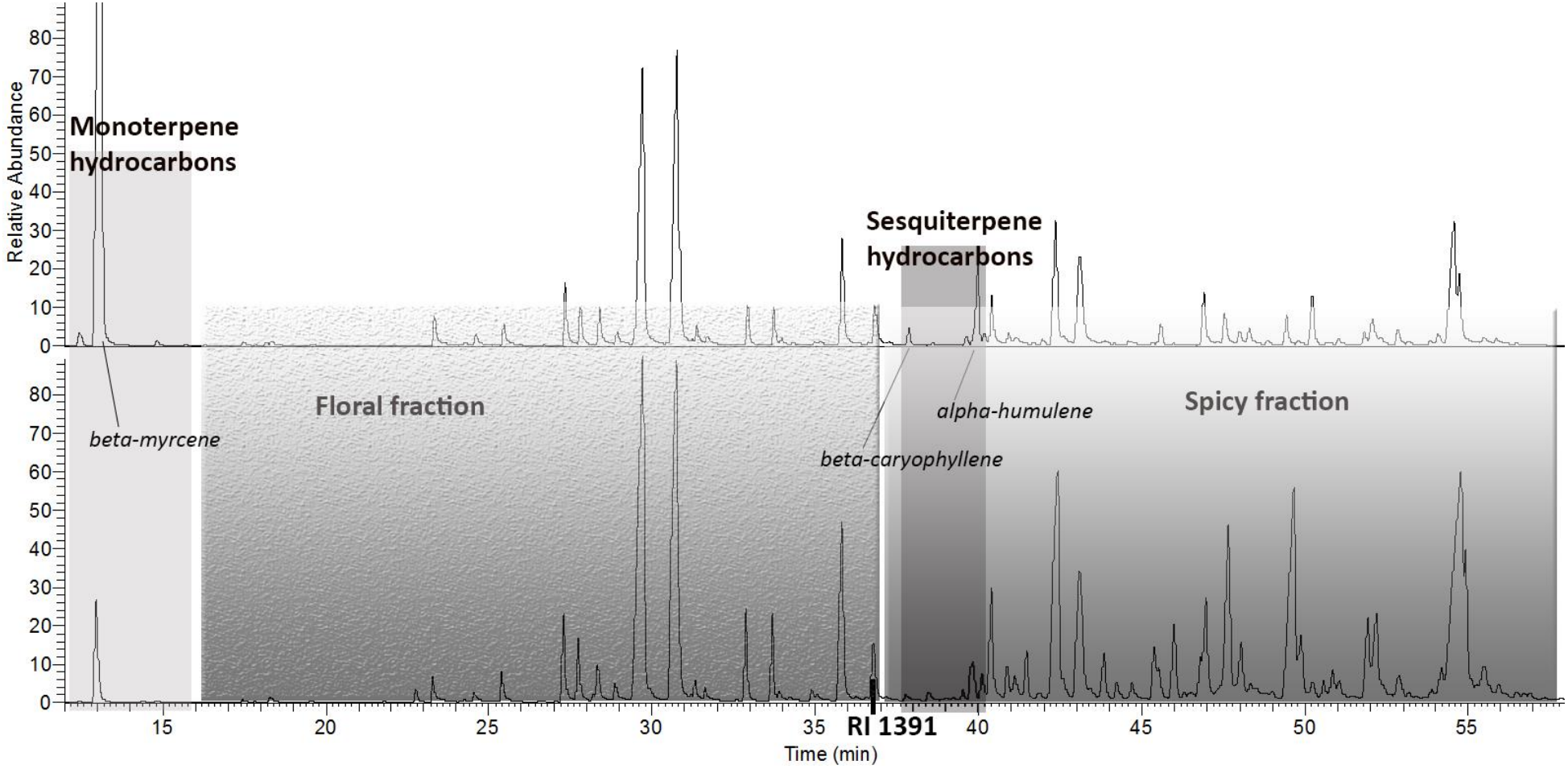
The SPE-fractions analysed via HS-SPME-GC-MS for determination of the reproducibility of the SPE procedure (**section 3.3.2**) were also subjected to comprehensive fingerprinting of the hop oil-derived volatile profile. Each fraction (U60, B60, U70, B70, U80 and B80) was analysed four times and volatiles were (tentatively) identified. Normalised peak areas provide information on the level of a particular compound, whereas relative peak areas (%) of volatiles represent their percentage within the total composition of the fraction under investigation. Recoveries of volatiles were calculated to compare quantities found in the boiled sample with quantities found in the corresponding unboiled sample. In the **Appendix A**, **B** and **C**, the composition of the SPE fractions (both U and B) eluting with 60% EtOH, 70% EtOH and 80% EtOH, respectively, is given. If for a particular compound no normalised and relative peak areas are given, this points to co-elution with (an) other compound(s) and a low peak area relative to those of co-eluting compounds. Considering all the fractions, between 3% (U80) and 21% (U60) of the total peak area is attributed to volatiles for which no structural information could be gained. For most of these volatiles the mass spectrometric detection results in unclear mass spectra, due to low levels and, consequently, low peak heights (close to baseline). In addition, several compounds that could not be identified (due to lack of reference compounds and comprehensive mass spectral libraries)

depicted mass spectra from which some information on the structure could be deduced. For example, many unidentified compounds with a MW of 220 were detected, suggesting that it concerns oxygenated sesquiterpenoids ($C_{15}H_{24}O$).

The compounds were subdivided into different groups according to their chemical structure and functional groups. These groups include esters (methyl esters, ethyl esters and other esters), aliphatic carbonyl compounds (aldehydes and ketones), aliphatic alcohols, monoterpene hydrocarbons, oxygenated monoterpenoids and their derivatives (alcohols, ketones, epoxides, ethers, esters and unidentified (= not further specified)), SHCs, OSs (alcohols, ketones, aldehydes, epoxides and unidentified), pyrans and furans, and unknown compounds. For each of these groups, the total normalised and relative peak area can also be found in the tables (see **Appendix A**, **B** and **C**). In addition, in accordance with **Chapter 2**, oxygenated compounds were also classified into a floral and spicy fraction on the basis of the chromatographic region in which they elute. The first eluting compound which depicts a mass spectrum that clearly points to an OS structure, is found in B80 at RI 1391. Oxygenated compounds eluting before RI 1391 were classified as floral compounds, whereas oxygenated volatiles with an RI greater than 1391 were classified as spicy compounds. This classification is further visualised in **Figure 3-4**, depicting the chromatograms of U70 and B70.

Figure 3-4. HS-SPME-GC-MS TIC (total ion) chromatogram of the SPE-derived fractions U70 and B70.

U70 (upper chromatogram)= fraction eluting with 70% EtOH upon SPE-fractionation of unboiled hop oil cv. Saaz. B70 (lower chromatogram)= fraction eluting with 70% EtOH upon SPE-fractionation of boiled hop oil cv. Saaz. Classification of hop oil-derived volatiles is based on of their chemical structure and their position in the HS-SPME-GC-MS chromatogram: monoterpene hydrocarbons, sesquiterpene hydrocarbons, and oxygenated compounds eluting before RI 1391 (=t_R 36.8) are classified into the floral fraction; oxygenated compounds eluting after RI 1391 are grouped as spicy compounds. Identical intensity scale to allow for direct comparison of chromatograms.



3.3.3.1 Comparison of U60 and B60

When comparing the composition of U60 with B60, one can conclude that qualitative differences are rather limited: a few compounds are characteristic for the unboiled samples (**Appendix A**, n°25, n°36, n°44, n°60 and a series of unknown volatiles), whereas 5 compounds were only detected in the boiled samples. It concerns 2 monoterpenoid derivatives (unknown monoterpenoids at RI 1062 (n° 28) and linalyl ethyl ether (n° 30)), 2 oxygenated sesquiterpenoids ($\Delta^{2,3}$-5α,8α-epoxy-caryophyllane (n° 39) and an unknown oxygenated sesquiterpenoid at RI 1533 (n° 41)), next to a pyran-derivative (n°62). Linalyl ethyl ether was identified in the previous chapter as a compound formed *de novo* upon boiling of total hop essential oil cv. Saaz.

Differences between unboiled and boiled fractions mostly consist of quantitative differences. The difference in the amount of humulol (n° 48) is most striking, as levels in B60 were up to 15 times higher compared to U60. Clearly, oxygenated compounds make up the largest part of the fractions (93% of the total peak area in U60 and 97% in B60). In both unboiled and boiled samples carbonyl compounds are predominant (37% and 35% for U60 and B60, respectively), followed by esters for U60 (21%) and OSs for B60 (27%). When comparing boiled samples versus unboiled samples, monoterpene and SHCs levels, as well as levels of floral and spicy compounds, are lower in the boiled samples (recovery of 9%, 65%, 81% and 93%, respectively). However, OS levels are clearly elevated in the boiled samples (recovery of 209%).

3.3.3.2 Comparison of U70 and B70

In general, the composition of U70 versus B70 samples shows much more qualitative differences compared to U60 versus B60 samples. Compounds exclusively found in unboiled samples comprise the oxygenated compounds n°25, 33 and 78 (**Appendix B**), the sesquiterpene hydrocarbons n°50-53 and, several unknown volatiles.

On the other hand, p-cymene (n°38), linalyl ethyl ether (n°42), terpinyl ethyl ether (n°43), and a series of unknown volatiles were only detected in the B70 samples. Linalyl ethyl ether, terpinyl ethyl ether, and the unknown volatile (n°96) were previously found upon boiling of total hop essential oil (cv. Saaz) (see **Chapter 2**).

In addition, a large series of OSs is found in the boiled B70 sample, whereas many of these compounds are not present in the unboiled U70 sample. Amongst these volatiles (n°54-56, 58, 60-67, 70-71, 74 and n°89), the unknown oxygenated sesquiterpenoid at RI 1428 (n°56), 1,5,8,8,-tetramethyl-12-oxa-5-tricyclo[7.2.1.$0^{6,9}$]dodecene (n°58), 4S-dihydrocaryophyllene-5-one (n°63), an unidentified oxygenated sesquiterpenoid at RI 1535 (n°66), and humulol (n°74) were previously indicated as newly formed upon boiling (see also **Chapter 2**). Nevertheless, low levels of the latter compound were detected in fraction U60 of the current experiment (see **Appendix A**), indicating that humulol is present in unboiled hop oil at levels close to the detection limit. Therefore, SPE fractionation proves to be a useful technique to

detect such compounds that would otherwise not be detected due to a combination of low levels and co-elution with other volatiles.

Monoterpene hydrocarbon levels are clearly higher in U70 (27%) compared to U60 (4%). In addition, levels of monoterpene hydrocarbons show a significant decrease upon boiling, which is reflected by the low levels found in B70 (3%). Similar to U60 and B60, U70 and B70 contain low levels of SHCs and high levels of carbonyl compounds (24% and 28% for U70 and B70 respectively), esters (21% and 19% for U70 and B70 respectively) and OSs, particularly in the boiled samples (27%). In general, it can be concluded that oxygenated compounds are predominant (70% for U70 and 96% for B70) and that their levels are higher in the boiled samples compared to their unboiled counterparts (recovery of 140% and 260% for resp. floral and spicy compounds in B70 compared to U70). When considering the OSs in particular, levels appear to be much higher in the boiled samples (recovery of 480%) whereas terpene levels are clearly lower (recovery of 15% and 42% for resp. mono- and sesquiterpene hydrocarbons).

3.3.3.3 Comparison of U80 and B80

Qualitative differences between the volatile profile of U80 and B80 cover compounds only detected in the unboiled samples (**Appendix C**, n°12, 13, 42, 49, 50, 54-57, 61, 63, 79, 94, 97, 99 and n° 113) and compounds that are specific for the boiled samples (n°9, 10, 26, 33-38, 43, 45, 67-72, 80, 83, 87, 92, 98, 104, 106-111, 115-117 and n° 121). Once more, we can see confirmation of the results obtained in **Chapter 2**, since linalyl ethyl ether (n°34), terpinyl ethyl ether (n°36), an unknown sesquiterpene hydrocarbon (n°37), (1R,8R,9S)-5,8-cyclocaryophyll-4-ene (n°38), *cis*-α-bergamotene (n° 43), 1,5,8,8,-tetramethyl-12-oxa-5-tricyclo[7.2.1.$0^{6,9}$]dodecane (n° 69), 4S-dihydro-caryophyllene-5-one/6(5→4)-abeo-8,12-cyclo-caryophyllan-5-al (n°71), 4,8,11,11-tetramethyl-8-tricyclo-[7.2.0.$0^{2,5}$]undecen-4-ol (n°80), two unknown oxygenated sesquiterpenoids (n°68, 87) and an unknown volatile (n° 98) were all previously found as newly formed compounds upon boiling of total hop oil cv. Saaz (**Chapter 2**).

In contrast with the SPE-fractions eluting with lower EtOH concentrations, U80 and B80 predominantly consist of SHCs (73% and 45% respectively). Monoterpene hydrocarbons reached a maximum level in U70 (26%), although also in U80 significant levels of this compound class can be found (15%). Due to high levels of terpene hydrocarbons, the relative portion of oxygenated compounds in U80 and B80 is significantly reduced. However, when considering normalised peak areas, the level of spicy compounds in U80 and B80 is only slightly lower than in U70 and B70 and significantly higher than in U60 and B60. Levels of floral compounds clearly reach a minimum in U80 and B80 compared to the other fractions.

3.3.3.4 Compounds formed *de novo* during boiling

It can be concluded that when applying SPE-fractionation of unboiled and boiled total hop oil (cv. Saaz) and subsequent HS-SPME-GC-MS analysis, a highly detailed analytical characterisation of the volatile profile of these hop oils can be obtained. Some compounds

were found to be specific for particular boiled SPE fractions. Nevertheless, in some cases, the same compound was found in an unboiled SPE fraction eluting with a different EtOH-concentration. Thus, some compounds that were pinpointed in **Chapter 2** as 'newly formed upon boiling', as was the case for *e.g.* humulol and 4S-dihydrocaryophyllene-5-one, may actually already be present in unboiled hop oil, but may not be detected when analysing total hop oil because of co-elution with other volatiles and low levels close to the spectrometer's detection limit. On the other hand, SPE-fractionation allows for determination of additional volatiles formed *de novo* during the boiling process. To summarise, next to the volatiles already indicated in **Chapter 2**, the compounds listed in **Table 3-2** are formed *de novo* upon boiling since these compounds were not detected in U60, U70 and U80.

Table 3-2. Additional compounds (next to Table 2-3) characteristic for boiled fractions derived from SPE-fractionation of total hop essential oil. Compounds are formed *de novo* during the boiling process. X marks the presence of the compound in the particular SPE fraction. RI= retention index.

		60% EtOH		70% EtOH		80% EtOH	
Volatile	RI	U60	B60	U70	B70	U80	B80
2H-2-Ethenyl tetrahydro-2,6,6-trimethyl-pyran	<1000	-	X	-	-	-	-
p-Cymene	1013	-	-	-	X	-	X
Fenchyl ethyl ether	1106	-	-	-	-	-	X
Terpinyl ethyl ether (isomer)	1245	-	-	-	-	-	X
4α,8α-Epoxy-caryophyllane	1402	-	-	-	X	-	X
4β,8β-Epoxy-caryophyllane	1408	-	-	-	X	-	-
Caryophylla-4(12),8(13)-diene	1414	-	-	-	-	-	X
$\Delta^{2,3}$-5α,8α-Epoxy-caryophyllane	1496	-	X	-	X	-	X
Unknown (m/z 69, 163)	1501	-	-	-	X	-	-
Unknown oxygenated sesquiterpenoid (MW 220)	1502	-	-	-	X	-	-
Unknown oxygenated sesquiterpenoid (mass spectrum highly similar to mass spectrum of 4,8,11,11-tetramethyl-8-tricyclo-[7.2.0.0^{2,5}]-undecen-4-ol)	1516	-	-	-	X	-	-
4R-Dihydrocaryophyllene-5-one	1528	-	-	-	X	-	-
6(5→4)-Abeo-caryophyll-7-en-5-al	1532	-	-	-	X	-	X
Unknown (m/z 69)	1584	-	-	-	X	-	-
Unknown oxygenated sesquiterpenoid (m/z 79, 93 ,105, 119, 220)	1597	-	-	-	-	-	X
Unknown (m/z 80, 93, 112, 121, 136)	1626	-	-	-	-	-	X

3.3.3.5 Quantification of volatiles in SPE-fractions of unboiled and boiled hop essential oil

Since the **Appendices A-C** do not provide quantitative information on the volatiles in the fractions, calibration curves (range 0-250 µg/L) of different reference compounds were drawn up as described in **section 3.2.5** in order to calculate exact concentrations. **Table 3-3** summarises the slope (a), intercept (b) and regression coefficient (R^2) of these curves. For most of the compounds, the linear calibration curve is characterised by highly satisfying regression coefficients. However, for 2-nonanone, this coefficient is significantly improved when a quadratic calibration curve is used (characterised by a, b and c). The resulting concentrations (in µg/L) can be found in **Table 3-3**.

Table 3-3. Calibration curves of reference compounds (0-250 µg/L) for quantification in SPE-derived fractions. t_R= retention time (min). Linear curves: a (slope) and b (intercept) are shown. Quadratic curves: a, b and c are shown. R^2= regression coefficient. D.L.= detection limit (µg/L).

Reference compound	t_R	a	b	c	R^2	D.L. (µg/L)	U50 (µg/L)	U60 (µg/L)	U70 (µg/L)	U80 (µg/L)	B50 (µg/L)	B60 (µg/L)	B70 (µg/L)	B80 (µg/L)
Ethyl isovalerate	7.91	0.0029	-0.0002	-	0.9953	23	0	0	0	0	1996	285	0	0
β-Pinene	12.40	0.0603	-0.0022	-	0.9948	1	0	34	444	876	0	0	82	218
β-Myrcene	12.94	0.0546	-0.0037	-	0.9908	1	104	2191	14420	30363	107	189	4317	6359
3-Carene	13.94	0.0412	-0.0006	-	0.9818	1	14	33	0	0	13	34	0	0
Methyl heptanoate	14.10	0.0175	-0.0032	-	0.9918	23	546	326	0	0	544	1170	0	0
Limonene	14.74	0.0823	0.0002	-	0.9965	1	10	16	98	359	0	0	47	348
γ-Terpinene	16.17	0.1036	-0.0023	-	0.9973	1	17	65	31	22	20	58	23	15
2-Nonanone	17.42	0.0005	0.0067	-0.0034	0.9989	24	386	708	311	224	503	658	270	228
Terpinen-4-ol	22.55	0.0025	-0.0006	-	0.9820	44	0	0	0	189	0	0	0	0
2-Decanone	23.23	0.0524	-0.0084	-	0.9939	5	265	1297	768	0	253	1302	592	0
Methyl nonanoate	25.39	0.2212	-0.0312	-	0.9935	46	0	112	205	0	0	84	198	0
2-Undecanone	29.51	0.1654	-0.0112	-	0.9995	5	151	1804	3551	382	61	1193	4570	498
Methyl decanoate	32.62	0.3444	-0.0069	-	0.9998	4	0	0	41	23	0	0	52	33
Geranyl acetate	34.88	0.0969	-0.0107	-	0.9981	1	0	57	94	0	0	53	138	77
2-Dodecanone	35.83	0.3889	-0.0129	-	0.9998	5	20	123	486	162	0	30	678	245
trans-Caryophyllene	37.95	0.7686	0.0233	-	0.9990	1	0	0	10	1338	0	0	0	381
α-Humulene	40.07	0.8641	0.0215	-	0.9984	1	0	34	113	9534	0	12	45	1280
trans-β-Farnesene	40.31	1.1915	0.0242	-	0.9998	1	0	01	0	1014	0	1	18	444
2-Tridecanone	42.46	0.6770	0.0002	-	0.9999	1.1	2	62	448	597	0	34	780	1096
Nerolidol	47.17	0.0153	-0.0019	-	0.9969	5	0	213	499	0	0	70	628	0

For specific quantification of linalool and geraniol, the method described in **section 3.2.6** making use of an ion trap mass spectrometer, was followed. This method for quantification of monoterpene alcohols has been developed by Van Opstaele[15].

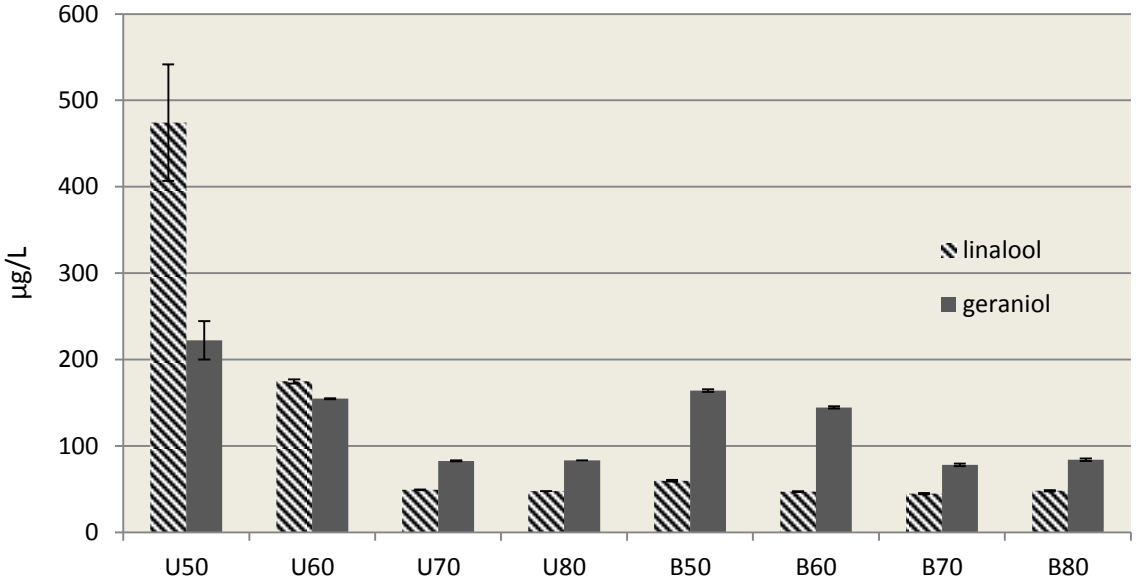


Figure 3-5. Levels of linalool and geraniol in SPE-derived fractions (obtained by SPE fractionation of total unboiled and boiled hop essential oil cv. Saaz). Levels are expressed in µg/L. Error bars represent standard deviation (n=2).

Using calibration curves with a correlation coefficient of 0.9994 and 0.9906 for linalool and geraniol respectively, levels of linalool and geraniol in the SPE fractions were quantified (see **Figure 3-5**). Clearly, linalool and geraniol reach maximal quantities in fractions eluting with 50% EtOH. With increasing EtOH concentration, lower levels of these monoterpene alcohols are found. Linalool levels are much lower in the boiled B50 and B60 fractions compared to their unboiled counterparts. For geraniol, this effect is much less pronounced, leading to relatively high levels in boiled fractions.

3.3.4 Sensory analysis of hop oil-derived SPE fractions via descriptive tasting tests and GC-olfactometry

3.3.4.1 Sensory evaluation of the SPE fractions in beer by a taste panel

SPE fractions eluting with 50, 60, 70 and 80% EtOH were spiked in non-aromatised beer (exclusively hopped with iso-α-acids CO_2 extract, aiming at a level of 25 ppm iso-α-acids), whereupon the beers were sensorially evaluated by our taste panel (for more details, see **section 3.2.8**).

In total, 4 sensory evaluations were carried out, during which beers with addition of U50 were compared to B50, U60 to B60, U70 to B70, and U80 to B80, respectively. The results of the descriptive tests are summarised in spider plots in **Figure 3-6** (**a**,**b**,**c** and **d**).

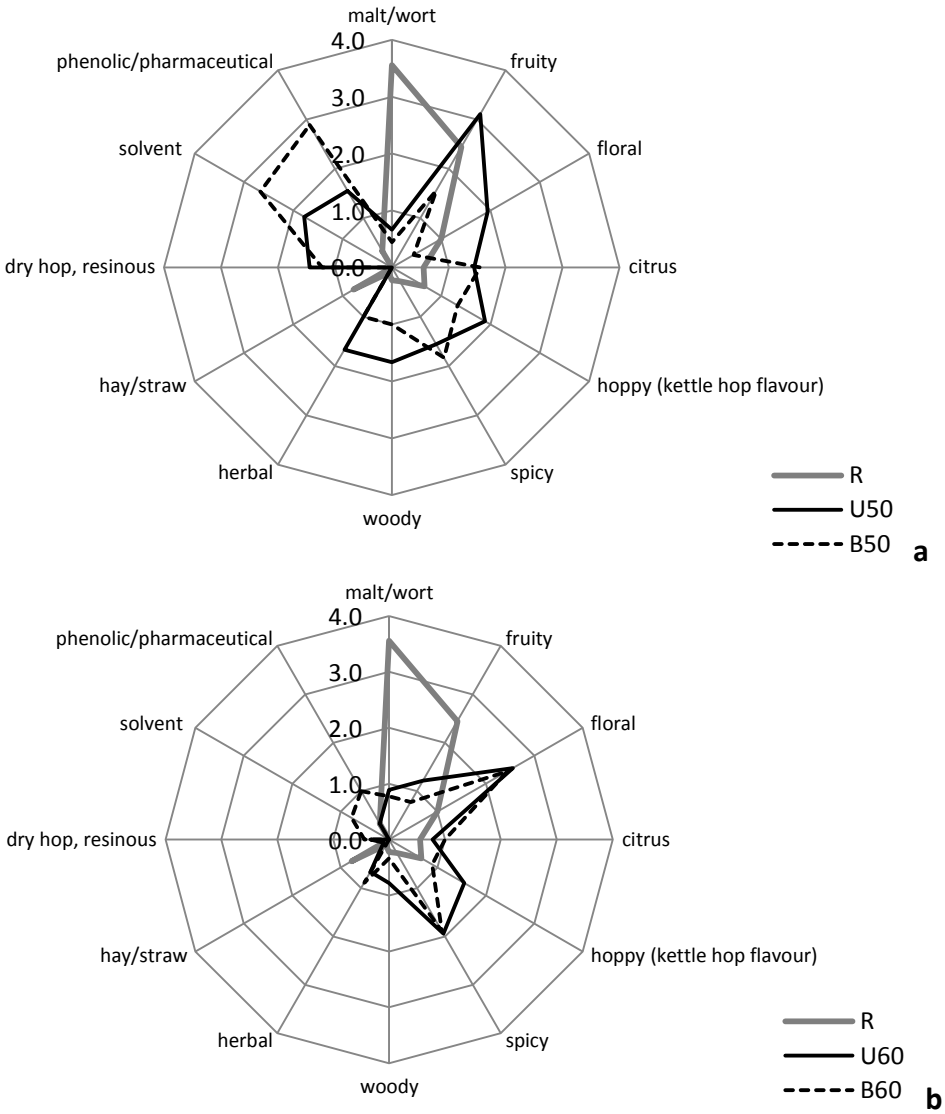


Figure 3-6. Spider plots representing scores for different descriptors used by the taste panel (12 panellists) to describe the flavour of non-aromatised iso-α-bittered lager beers spiked with U50 vs. B50 (a) and U60 vs. B60 (b) fractions. Fractions derived upon SPE fractionation of unboiled and boiled total hop essential oil (cv. Saaz). R= reference beer without addition.

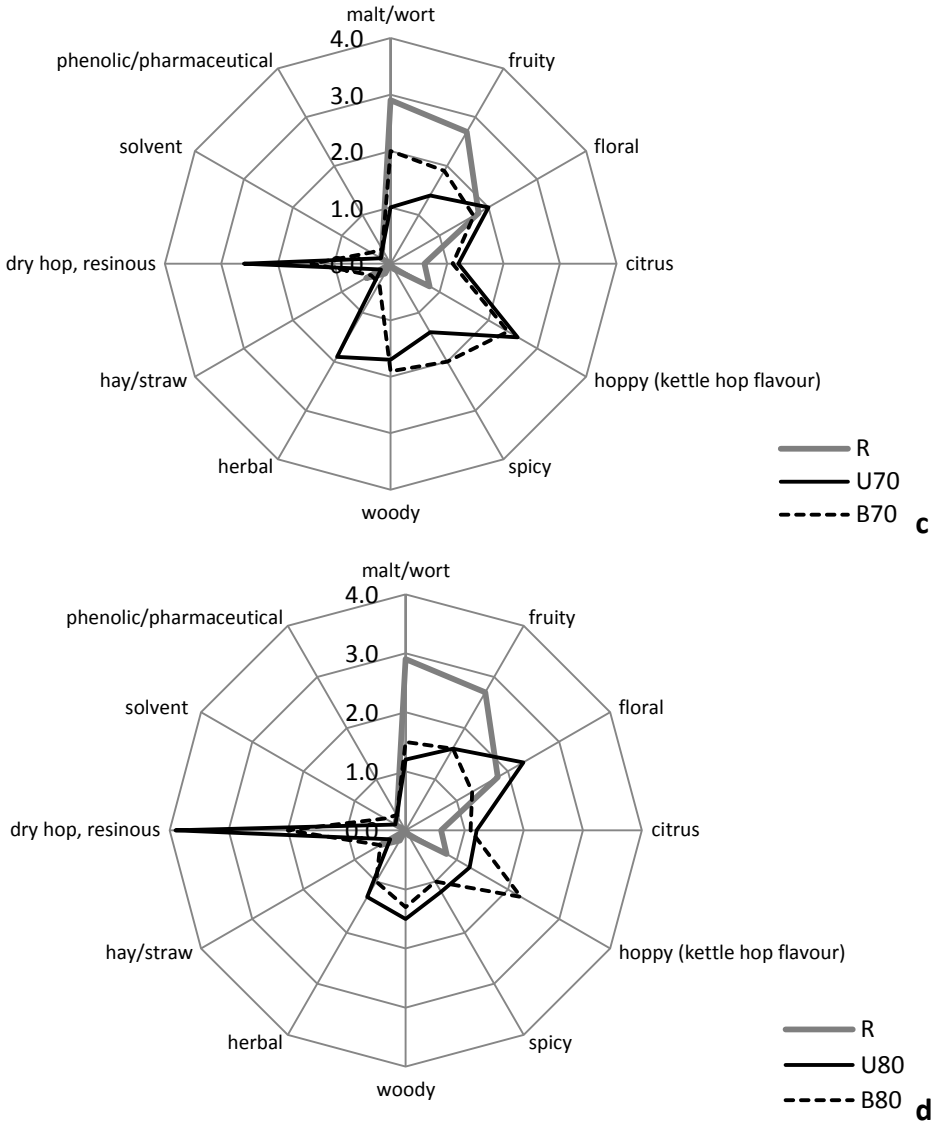


Figure 3-6. Spider plots representing scores for different descriptors used by the taste panel (12 panellists) to describe the flavour of non-aromatised iso-α-bittered lager beers spiked with U70 vs. B70 (c) and U80 vs. B80 (d) fractions. Fractions derived upon SPE fractionation of unboiled and boiled total hop essential oil (cv. Saaz). R= reference beer without addition.

The reference beer is mainly characterised by 'malty/worty' and 'fruity' flavours.

In **Figure 3-6 a**, the flavour profiles of the beers with addition of U50 and B50 are given. The beer with addition of U50 was described as 'fruity', 'floral', 'hoppy', 'herbal' and 'woody'. The relatively high score for 'floral' in U50 compared to B50 might be ascribed to significant lower linalool levels in B50 (see **section 3.3.3**) Also 'solvent' and 'phenolic' notes were perceived. However, the beer with addition of B50 was scored much higher for the latter descriptors, which confirms the results obtained in **section 3.3.1**.

The beers with addition of the 60% EtOH fractions (both U60 and B60) are scored relatively high for 'floral' and 'spicy' notes (**Figure 3-6 b**), which can be related to the analytical results. These fractions are mainly composed of 'floral' and 'spicy' compounds (see **section 3.3.3**).

Although U60 contains higher levels of linalool compared to B60, both fractions lead to a comparable intensity of 'floral' notes when added to beer. Solvent-like flavours are slightly elevated in B60, although scores are much lower compared to the beer with addition of B50. Upon addition of U70, B70, U80, and B80, respectively, these off-flavours are not detected.

'Dry hop/resinous' and 'herbal' top notes are characteristic for the flavour profile of the beer with addition of U70 (**Figure 3-6 c**). The expression of this odour might be attributed to fairly high β-myrcene levels in U70 (14,420 µg/L). β-myrcene has been proposed as a key character impact compound for the aroma of 'fresh hops'[4,122]. In the beer with addition of B70, these specific flavour notes are much less pronounced, which can be explained by significantly lower β-myrcene levels in B70 (4,317 µg/L). Scores for 'floral', 'citrus' and 'hoppy' notes are comparable for the beers spiked with U70 and B70, whereas the elevated OS levels in B70 compared to U70 might lie at the basis of somewhat more pronounced 'woody' and 'spicy' notes in the corresponding beer.

The high β-myrcene levels in U80 (30,364 µg/L) compared to U70 are reflected in the sensory results since beer showed even more pronounced 'dry hop/resinous' notes upon addition of B80 (**Figure 3-6 d**). Moreover, B80 also contains high SHC levels (1,338 µg/L β-caryophyllene, 9,534 µg/L α-humulene and 1,014 µg/L β-farnesene), which may also be potent contributors to a 'resinous' hop aroma[165]. Both monoterpene and sesquiterpene hydrocarbon levels are clearly lower in B80. Although beer spiked with B80 was also scored much lower for 'floral' top notes and slightly lower for 'herbal', 'woody' and 'spicy' odours, the score for 'kettle hop' flavour is elevated.

It can be concluded that the results obtained by the sensory panel are generally comparable to those obtained during the preliminary sensory evaluation (see **Table 3-1**). B60 imparts somewhat weaker flavours compared to B50, B70 and B80 upon addition to beer. The odour of B50 is characterised by off-flavours and thus considered as uninteresting regarding 'kettle hop aroma'. However, the fractions B70 and B80 do show potential to contribute to 'kettle hop flavour'. Compared to U70 and U80, addition of fraction B70 to beer increases 'woody' and 'spicy' notes, and elevated scores for 'hoppy' were observed upon addition of B80. Moreover, the boiled fractions show less 'resinous' top notes, typical for the aroma of unprocessed hops.

3.3.4.2 Determination of flavour-active compounds in SPE fractions via GC-MS/O

Taking into consideration the interesting flavours perceived during sensory evaluation of the SPE fractions in beer, the fractions were analysed via GC-MS/O (as described in **section 3.2.7**) to determine flavour-active intervals and volatiles in these intervals. The detection

frequency, which is the amount of times a particular flavour-active peak was detected out of the 6 analyses, provides an estimation of the significance of the perceived odour.

U60 vs B60

Table 3-4 shows the detection frequencies in flavour-active zones of U60 and B60, respectively. These fractions are characterised by some flavour-active compounds that were previously found in a floral hop essence cv. Spalter Select by Van Opstaele *et al.*[123]. Common odour-active volatiles are β-myrcene, nonanal, methyl nonanoate and 2-undecanone, which may contribute to the floral top note. Linalool levels in B60 are clearly lower compared to U60 (see **Figure 3-5**), which is also reflected in the detection frequencies (5 and 3 in U60 and B60, respectively).

A number of odour-active peaks consisted of co-eluting compounds (unclear mass spectra) and in 2 zones no chromatographic peak was detected, pointing to levels of the relevant odour-active compound below the mass spectrometric detection limit. One of these odour-active regions (RI 1222) was clearly more detectable in B60 than U60, which indicates increased (yet undetectable) levels in B60 compared to U60.

Table 3-4. Tentative identification (on the basis of MS and RI) of volatiles eluting in flavour-active regions upon GC-MS/O of U60 and B60. RI= retention index at start of odour-active interval. DF= detection frequency out of 6 analyses (3 trained assessors, duplicate analyses). Only compounds with DF≥3 (out of 12 analyses in total) are shown.

Tentative identification of volatiles in flavour-active regions	RI	DF U60	DF B60
β-Myrcene	<1000	2	1
Unknown (below detection limit)	1061	2	1
Nonanal	1081	2	1
Linalool	1085	5	3
Methyl 2-methyloctanoate	1152	2	1
2-Decanone	1180	2	3
Decanal	1189	3	1
Methyl nonanoate	1213	1	2
Unknown (below detection limit)	1222	1	4
5-Undecen-2-one	1258	3	1
2-Undecanone	1272	2	1
Unknown(unclear mass spectrum, co-elution)	1360	1	2
Unknown(unclear mass spectrum, co-elution)	1363	1	2
Unknown (m/z 79, 80, 81, 93, 122, 136, 164)	1394	2	1
Unknown(unclear mass spectrum, co-elution)	1396	1	2
β-Ionone	1456	1	5
Unknown (m/z 69, 81, 95, 109, 123, 138, 149, 205, 220)	1472	1	2
4S-Dihydrocaryophyllene-5-one	1521	1	3
4R-Dihydrocaryophyllene-5-one	1530	2	2
Clovenol	1560	3	1
Caryophylla-4(12),8(13)-diene-5-ol	1609	1	2
Cubenol/δ-cadinol	1618	1	2
3Z-Caryophylla-3,8(13)-diene-5α-ol	1628	5	1
Unknown (unclear mass spectrum, co-elution with unknown (m/z 79, 80, 81, 164, 222))	1627	4	5
3Z-Caryophylla-3,8(13)-diene-5β-ol	1627	2	2

Another compound that might have increased in its level upon boiling of hop oil is β-ionone, an oxidative degradation product of β-carotene and belonging to the norisoprenoid compound class. Although β-ionone was not detected during analytical profiling (see **section 3.3.3**), it was detectable in the GC-MS/O chromatograms. This compounds, detected in hops for the first time by Tressl and coworkers[82] is highly flavour-active (flavour threshold of 0.008 ppb[88]) and described as 'floral' and 'violet-like'[12]. Although this compound is present in beer at levels at which it may be an important contributor to hoppy aroma of beer[147], Lermusieau and Collin[7] did not detect β-ionone upon GC-O analysis of beer and therefore stated that it probably does not influence hoppy character.

The compounds discussed above are likely responsible for the 'floral' odour perceived by addition of U60 and B60 to non-aromatised iso-α-acid bittered beer, whereas the 'spicy' flavour might be attributed to flavour-active OSs, such as caryophyllene derived ketones and alcohols.

U70 vs. B70

In **Table 3-5**, the results of the GC-O analyses on U70 and B70 are summarised. The higher odour intensity of the SPE fractions eluting with 70% EtOH compared to 60% EtOH observed during sensory evaluation of the beers spiked with these fractions, is clearly expressed by the high number of odour-active intervals.

β-Myrcene reached a maximum detection frequency in U70 which dropped to 3 in B70. The unboiled fraction is indeed characterised by high β-myrcene levels and was found to impart 'resinous' and 'dry hop' aroma upon addition to beer. Compounds such as nonanal and 2-undecanone, previously found in a floral hop oil-derived essence[123] as well as in the U60 and B60 samples, and described as 'citrus' and 'floral'[123], also eluted in flavour-active intervals of the U70 and B70 fractions. In addition to these volatiles, methyl octanoate and 2-dodecanone also expressed flavour in both our fractions.

Although several SHCs also expressed odour, OSs are clearly making up the major part of hop oil-derived volatiles detected in flavour-active intervals. Moreover, many of these compounds proved to be oxidation products formed *de novo* upon boiling. Therefore, formation of such odour-active OSs during real wort boiling might be the reason why 'early' kettle hopped beers are characterised by 'spicy/herbal' notes. Several of these compounds were also detected upon GC-O analyses of U60 and B60 (4S-dihydrocaryophyllene-5-one, 4R-dihydrocaryophyllene-5-one, caryophylla-4(12),8(13)-diene-5-ol, (3Z)-caryophylla-3,8(13)-diene-5α-ol and (3Z)-caryophylla-3,8(13)-diene-5β-ol) and are again detected in the 70% EtOH fractions. In addition, 1,5,8,8-tetramethyl-12-oxa-5-tricyclo[7.2.1.$0^{6,9}$]dodecene, $\Delta^{2,3}$-5α,8α-epoxy-caryophyllane and 6(5→4)-abeo-caryophyll-7-en-5-al, characteristic for boiled

hop oil, were only detected in the boiled fraction. Humulene epoxide III and humulenol II may be potent odour-active compounds, since they both were detected 4 and 5 times out of the 6 analyses in U70 and B70, respectively.

Table 3-5. Tentative identification (on the basis of MS and RI) of volatiles eluting in flavour-active regions upon GC-MQ/O of U70 and B70. RI= retention index at start of odour-active interval. DF= detection frequency out of 6 analyses (3 trained assessors, duplicate analyses). Only compounds with DF≥3 (out of 12 analyses in total) are shown.

Tentative identification of volatiles in flavour-active regions	RI	DF U70	DF B70
β-Myrcene	<1000	6	3
Unknown (below detection limit)	1034	2	2
trans-β-Ocimene	1041	2	1
Nonanal	1085	2	1
Linalool	1085	1	2
Methyl octanoate / methyl 2,6-dimethylheptanoate	1111	3	3
Unknown (below detection limit)	1143	2	2
Methyl 2-methyloctanoate	1148	1	4
2-Decanone	1175	1	3
Methyl 3-nonenoate	1194	0	3
Methyl nonanoate	1209	0	3
5-Undecen-2-one	1260	3	1
2-Undecanone	1274	4	3
Methyl *trans*-4-decenoate	1293	4	2
Unknown (unclear mass spectrum)	1296	2	1
Unknown (unclear mass spectrum)	1318	1	2
α-Ylangene	1372	2	2
2-Dodecanone	1380	2	1
Unknown (m/z 79, 80, 81)	1392	1	2
Unknown (below detection limit)	1404	2	2
β-Caryophyllene	1408	3	1
Unknown oxygenated sesquiterpenoid (m/z 69, 81, 95, 109, 123, 138, 149, 191, 205, 220)	1436	3	2
β-Farnesene	1442	2	1
5-Tridecen-2-one	1446	1	2
Unknown oxygenated sesquiterpenoid (m/z 91, 105, 119, 131, 146, 159, 177, 187, 202, 220)	1457	2	3
γ-Muurolene	1460	3	0
1,5,8,8,-Tetramethyl-12-oxa-5-tricyclo[7.2.1.$0^{6,9}$]dodecene	1462	0	3
Unknown oxygenated sesquiterpenoid (m/z 69, 81, 95, 109, 123, 138, 149, 191, 205, 220)	1468	3	0
$\Delta^{2,3}$-5α,8α-Epoxy-caryophyllane	1490	0	3
δ-Cadinene	1505	2	1
(4S)-Dihydrocaryophyllene-5-one / 6(5→4)-abeo-8,12-cyclo-caryophyllan-5-al	1536	2	3
(4R)-Dihydrocaryophyllene-5-one	1536	0	4
Sesquiterpene alcohol	1536	1	4
6(5→4)-Abeo-caryophyll-7-en-5-al	1536	0	4
Unknown oxygenated sesquiterpenoid (m/z 93, 205, 220) / unknown (m/z 79, 80, 81, 150)	1540	1	4
6(5→4)-Abeo-caryophyll-8(13)-en-5-al	1552	3	2
Humulene epoxide III	1601	4	5
Humulenol II	1601	4	5
Caryophylla-4(12),8(13)-diene-5-ol	1601	1	4
τ-Cadinol	1601	2	3
(3Z)-Caryophylla-3,8(13)-diene-5α-ol	1630	4	3
Unknown (unclear mass spectrum, co-elution with unknown (m/z 79, 80, 81, 164, 222))	1630	6	2
Unknown	1630	1	2
(3Z)-Caryophylla-3,8(13)-diene-5β-ol	1630	4	2
Humulene allylic alcohol	1630	4	3
Unknown	1630	1	3
α-Bisabolol	1630	3	3

The GC-O results are clearly in line with previous analytical and sensory results: the B70 fraction was analytically characterised by high levels of 'spicy compounds' (see **section 3.3.3.2**) and imparted elevated 'woody' and 'spicy' odours compared to U70 upon addition to beer (see **Figure 3-6 c**).

U80 vs. B80

The U80 and B80 fractions are also characterised by many flavour-active zones, detected upon GC-O analysis (see **Table 3-6)**. β-Myrcene appears to be the most potent odour-active volatile as it was detected 6 times in both fractions. U80 indeed contains the highest β-myrcene levels of the investigated fractions. Moreover, also the boiled sample still contains relatively high amounts of this volatile, which explains the 'resinous' notes perceived by our taste panel upon spiking of lager beers with these fractions (see **Figure 3-6 d**). The monoterpene hydrocarbon *trans*-β-ocimene, which was detected in the U70 and B70 fractions (see **Table 3-5**), was also found in an odour-active zone during GC-O analysis of U80 and B80. This was also the case for methyl octanoate and 2-undecanone. Moreover, ethyl nonanoate, which was proven to be a 'fruity' odour impact compound of a Spalter Select-derived floral hop essence[123], was detected in both the U80 and B80 fraction.

The number of SHCs detected in flavour-active zones was rather limited, suggesting that their contribution to the 'resinous' and 'dry hop' flavour of these fractions is of less importance than the impact of β-myrcene.

Odour-active OSs might be responsible for 'kettle hop' aroma when B80 is added to non-aromatised lager beer. The unknown oxygenated sesquiterpene with RI 1541 was previously mentioned in **Chapter 2**, where it was indicated as a compound formed *de novo* upon boiling of total hop essential oil. This explains why the odour of this volatile was only perceived in B80.

Caryophyllene oxide also appears to elute in a flavour-active interval, and has been indicated as an odour-active compound of a spicy hop essence cv. Spalter Select by Van Opstaele and coworkers[124].

Similar to the U70 and B70 fractions, humulene epoxide III and humulenol II appear to elute in odour-active zones within the U80 and B80 fractions.

Cubenol and τ-cadinol were previously found in odour-active zones of the fractions that eluted with 60% EtOH and 70% EtOH, respectively.

Also worth mentioning is (3Z)-caryophylla-3,8(13)-diene-5α-ol, which was found in an odour-active zone of all the fractions investigated. (3Z)-caryophylla-3,8(13)-diene-5β-ol, however, did not appear in an odour-active zone in the fractions eluting with 80% EtOH . Nevertheless,

this compound was found in U60, U70, B60 and B70, and has been proposed as a key odour impact compound of hop teas and ale beer[146].

Finally, special attention should be paid to an unknown volatile (RI 1628) that was clearly detected in all the SPE-derived fractions. This compound's mass spectrum is unclear, due to co-elution with a (from a quantitative point of view) major volatile characterised by distinct mass fragments at m/z 79, 80, 81, 164 and 222. Nevertheless, when looking at detection frequencies, this unknown volatile might have a major impact on the flavouring characteristics of our SPE-fractions.

Table 3-6. Tentative identification (on the basis of MS and RI) of volatiles eluting in flavour-active regions upon GC-MQ/O of U80 and B80. RI= retention index at start of odour-active interval. DF= detection frequency out of 6 analyses (3 trained assessors, duplicate analyses). Only compounds with DF≥3 (out of 12 analyses in total) are shown.

Tentative identification of volatiles in flavour-active regions	RI	DF U80	DF B80
β-myrcene	<1000	6	6
trans-β-Ocimene	1043	3	1
Unknown (m/z 69, 109, 123)	1075	1	2
Methyl octanoate / methyl 2,6-dimethylheptanoate	1110	2	1
Unknown (below detection limit)	1148	2	1
Unknown (below detection limit, elution place of linalyl ether)	1164	2	1
Unidentified methyl ketone	1236	2	1
Ethyl nonanoate	1247	2	1
Methyl 4,6-dimethyloctanoate	1265	3	1
2-Undecanone	1270	2	3
Unidentified ester (m/z 88, 101)	1280	1	2
Methyl decanoate	1305	1	3
trans-α-Bergamotene	1429	4	0
α-Humulene	1440	2	3
trans-Calamenene	1503	2	2
Unknown (below detection limit)	1511	2	1
Unknown (below detection limit)	1540	1	4
Unkknown oxygenated sesquiterpenoid (m/z 93, 137, 205, 220)	1541	0	3
Caryophyllene oxide	1554	2	1
Humulene epoxide I	1569	0	4
Unidentified methyl ketone	1574	1	2
Unknown (unclear mass spectrum)	1584	2	1
Humulene allylic alcohol	1587	1	2
Humulene epoxide III	1600	3	4
Humulenol II	1603	4	3
τ-Cadinol	1614	2	2
Cubenol	1619	3	1
(3Z)-Caryophylla-3,8(13)-diene-5α-ol	1628	4	1
Unknown (unclear mass spectrum, co-elution with unknown (m/z 79, 80, 81, 164, 222))	1628	4	4
Cadalene	1631	1	3
6Z-Pentadecen-2-one	1631	1	4
Humulene allylic alcohol	1648	2	2
Unknown	1648	2	3

3.3.5 GC-MS fingerprinting of the hop-derived OS spectrum of commercial kettle hopped lager beers

Investigation of the composition of the hop-derived oxygenated sesquiterpenoid (OS) fraction in beer is not straightforward and an analytical challenge on account of the high complexity of this particular fraction, the high risk of co-elution (hampering accurate identification) when performing monodimensional GC-MS, and the extremely low levels at which these constituents are present in beer. To tackle this problem, an SPE-based methodology was developed to extract hop oil constituents, in particular OSs, from beer and to enrich and distribute compounds of interest over different fractions. As discussed in **section 3.3.3**, the fractions eluting with 70% and 80% ethanol contain the highest level of OSs and, therefore, these two fractions were analysed via HS-SPME-GC-MS in order to characterise the OS profile of 3 commercial kettle hopped lager beers.

On the basis of calculated retention indices, in combination with mass spectra, compounds eluting in the OS chromatographic region were identified. The results are summarised in **Table 3-7**. In total, 63 different compounds were detected in the chromatographic region where OSs elute and, moreover, 43 compounds were (tentatively) identified. Clearly, 33 identified volatiles belong to the chemical class of OSs. In addition, 11 more compounds, although their precise identity could not be revealed, are proposed to be OSs on account of their mass spectra (*i.e.* typical fragmentation pattern and recognition of the molecular ion at m/z 218, 220 or 222). Thus, the results show that most of the detected compounds are OSs, proving that the proposed SPE based methodology allows for selective isolation of the OS hop oil fraction from beer. Apparently, this class of compounds (partially) survives the brewing process and therefore OSs are very important from an analytical point of view (cf. analytical fingerprinting of beer). Moreover, according to literature data, OSs are also considered to be important regarding sensory properties as they have been suggested to contribute to kettle hoppy aroma of beer[10,17–19,27,160,173]. Next to the OSs, we also detected 5 sesquiterpene hydrocarbons (n° 3, 9, 12, 13, 53) and 3 compounds do not show a terpene-derived chemical structure (n° 7, 8, 58).

Table 3-7. Compounds detected in the chromatographic region where the oxygenated sesquiterpenoids (OSs) elute (full scan detection (m/z 40-265) and subsequent extracted ion chromatograms (m/z 79, 91, 93, 105, 121, 131, 133, 149, 202, 205, 218, 220, 222)). Identifications based on match for both retenction index (RI) and mass spectrum (MS), except no. 4 (only match for MS[84,138]). [R]= verification of identity by pure reference compound. no.= peak number, t_R= retention time (min), *= OS (if the compound is unknown, the MS suggests an OS structure), x= detected in beer A, B and/or C. Compounds in **bold** are detected in all investigated beers.

no.	RI	t_R	compound	A	B	C
1	1436	42.77	**Unknown (m/z 69, 81, 95, 109, 123, 205, 220)***	**x**	**x**	**x**
2	1441	43.12	Unknown (m/z 135, 149, 163, 177, 205, 207, 220, 222)*	x		x
3	1443	43.28	β-Farnesene[R]		x	x
4	1464	44.86	**1,5,8,8,-Tetramethyl-12-oxa-5-tricyclo[7.2.1.0^{6,9}]dodecene***	**x**	**x**	**x**

Table 3-7 continued

5	1470	45.31	Unknown (m/z 69, 81, 95, 109, 123, 205, 220)*			x
6	1481	46.07	Unknown (m/z 69, 83, 121, 139)	x	x	
7	1488	46.6	**Ionol**	**x**	**x**	**x**
8	1490	46.73	**3,5-Di-tertylbutyl-phenol**	**x**	**x**	**x**
9	1493	46.97	δ-Cadinene	x	x	
10	1493	46.95	Unknown (m/z 177, 220)*	x		
11	1505	47.88	Iso-korajol*	x	x	
12	1516	48.82	***trans*-Cadina-1,4-diene**	**x**	**x**	**x**
13	1521	49.17	**α-Calacorene**	**x**	**x**	**x**
14	1527	49.66	4S-Dihydrocaryophyllene-5-one*	x	x	
15	1527	49.73	6(5→4)Abeo-8,12-cyclo-caryophyllan-5-al*	x	x	
16	1538	50.58	Unknown (79, 80, 81, 150, 157, 220)	x		
17	1538	50.58	Unknown oxygenated sesquiterpenoid (m/z 93, 205, 220)*		x	
18	1543	50.97	**(E)-Nerolidol*^{R}**	**x**	**x**	**x**
19	1548	51.39	**Caryolan-1-ol***	**x**	**x**	**x**
20	1548	51.44	**Humuladienone***	**x**	**x**	**x**
21	1553	51.85	6(5→4)-Abeo-caryophyll-8(13)-en-5-al*		x	
22	1560	52.42	**Clovenol***	**x**	**x**	**x**
23	1564	52.76	Unknown (m/z 107,135,161, 218)*	x	x	
24	1565	52.77	Gleenol*	x	x	
25	1572	53.42	**Humulene epoxide I***	**x**	**x**	**x**
26	1577	53.79	**Humulol***	**x**	**x**	**x**
27	1582	54.23	**Humulene epoxide II***	**x**	**x**	**x**
28	1589	54.76	**Unknown (m/z 81,123,135,161, 179, 189, 204, 207)***	**x**	**x**	**x**
29	1591	54.92	Humulene allylic alcohol*		x	
30	1593	55.1	**1,10-Di-*epi*-cubenol***	**x**	**x**	**x**
31	1596	55.32	Junenol*	x	x	
32	1600	55.65	Unknown (m/z 70)	x		x
33	1601	55.76	Unknown (m/z 59, 81, 135, 149, 161, 164, 179, 189, 204)*	x		
34	1604	56.00	**Humulene epoxide III***	**x**	**x**	**x**
35	1606	56.16	**1-*epi*-Cubenol***	**x**	**x**	**x**
36	1607	56.23	**Humulenol II***	**x**	**x**	**x**
37	1608	56.32	**Unknown (m/z 119, 161, 179, 189, 204)***	**x**	**x**	**x**
38	1611	56.6	Caryophylla-4(12),8(13)-diene-5-ol*		x	
39	1617	57.09	**τ-Cadinol***	**x**	**x**	**x**
40	1618	57.16	τ-Muurolol*			x
41	1621	57.45	**Cubenol***	**x**	**x**	**x**
42	1624	57.70	β-Eudesmol*	x	x	
43	1627	57.91	**Selin-11-en-4-α-ol***	**x**	**x**	**x**
44	1629	58.10	**α-Cadinol***	**x**	**x**	**x**
45	1632	58.39	(3Z)-Caryophylla-3,8(13)-diene-5α-ol*	x	x	
46	1634	58.54	Unknown (m/z 79, 80, 81,164, 222)	x	x	
47	1636	58.73	Unknown (m/z 79, 80, 81,162, 220)	x		
48	1638	58.90	**Unknown (m/z 119, 121, 191)**	**x**	**x**	**x**
49	1641	59.09	Intermedeol*	x		
50	1641	59.09	14-Hydroxy-β-caryophyllene*	x		
51	1644	59.41	Unknown (m/z 93,137)	x	x	
52	1644	59.41	Unknown (m/z 191)	x	x	
53	1646	59.50	Cadalene	x	x	
54	1647	59.60	(3Z)-Caryophylla-3,8(13)-diene-5β-ol*	x	x	
55	1649	59.77	Unknown (m/z 55, 69, 82, 93, 120, 138, 222)	x	x	
56	1654	60.19	Humulene allylic alcohol*	x	x	
57	1654	60.23	Unknown (m/z 79, 80, 81)		x	
58	1661	60.82	Tetradecanol	x	x	
59	1665	61.15	Unknown (m/z 69, 82)	x	x	
60	1667	61.34	Eudesm-7(11)-en-4α-ol*	x	x	
61	1671	61.66	Farnesal*		x	x
62	1686	62.90	Humulene diepoxide A*	x	x	
63	1698	63.93	Farnesol*		x	x

The major part of the hop-derived OS fraction consists of β-caryophyllene and α-humulene derivatives. These compounds are oxidation products from their parent SHC molecule, formed during ageing and possibly during the boiling of hops[10,16,19,39,113]. Nine compounds are clearly derived from β-caryophyllene (**Table 3-7**, n° 14, 15, 19, 21, 22, 38, 45, 50, 54), whereas 10 compounds are α-humulene derivatives (n° 4, 20, 25, 26, 27, 29, 34, 36, 56, 62). The chemical structure of compound n° 11, *i.e.* iso-korajol (see **Figure 3-8**)(IUPAC name: (1R,2R,5S,6R,9R)-2,6,10,10-tetramethyltricyclo[7.2.0.$0^{2,5}$]undecan-6-ol), suggests that this compound is derived from β-caryophyllene or α-humulene. This compound has not been reported in literature within the context of hops and beer, but its mass spectrum, retention index and chemical structure were reported by Tkachev[189].

Two β-farnesene derivatives (n° 61, 63) were detected in beer B and C. Trace levels of β-farnesene (n° 3) were also found in these beers, suggesting that they were brewed with a β-farnesene rich hop variety (*e.g.* cv. Saaz, Lublin, Styrie, Backa, Spalt, Tettnang or Hallertau[79,80,199]). This observation is also in accordance with data provided for beer B (see **section 3.2.3**), *i.e.* hop aromatisation using cv. Hallertau Mittelfrüh and cv. Tettnang Tettnanger.

Remarkably, all the other identified OSs not discussed above, except for nerolidol (n° 18) and gleenol (n° 24), depict a highly similar chemical structure ($C_{15}H_{26}O$) with a molecular mass of 222 and contain two 6-ring structures (having 2 adjacent carbon atoms in common), one hydroxyl group and one unsaturated bond (for examples of such structures, see **Figure 1-9**, structures **(19)**-**(23)**). Particular compounds of this group, such as the cadinols (derived from the cadinane skeleton) tend to have a higher concentration in noble aroma hops and are probably not formed by chemical oxidation of SHCs but instead related to the hop plant biosynthesis[18]. Although clearly different from the β-caryophyllene and α-humulene oxidation products, these compounds (cadinols but also muurolols, eudesmols, cubenols, etc.) might be important with respect to hoppy aroma, since they clearly survive the brewing process and are detected in the beers investigated in our study. Furthermore, τ-cadinol (n° 39) has been proposed in literature as a contributor to hoppy aroma[77,139].

Clearly, despite interference from fermentation fusel alcohols and esters, the relatively low level of hop-derived volatiles in beer and co-elution of various compounds, a large series of OSs has been detected and identified in the volatile profile of lager beer using SPE-enrichment and fractionation. Amongst them, some compounds have already been identified in hops and beer decades ago (**Table 3-7**, *e.g.* n° 18, 19, 20, 25, 26, 27, 34, 35, 36, 39, 41, 42, 43, 44, 62, 63)[10,16–18,28,78,83,88,113,139,160,196,199], whereas the presence of other compounds was only recently confirmed in hops (*e.g.* n° 24, 50)[114,124,200] and/or beer (*e.g.* n° 24, 30, 38, 49, 50, 54)[146,197].

The main caryophyllene oxidation product, *i.e.* caryophyllene oxide, was not detected in the beers in our study, which is in agreement with previous findings[10,28] and can be attributed to the fact that this compound is prone to hydrolysis and isomerisation reactions[28,85,174]. Interestingly, some caryophyllene oxide derived compounds were already found in hop essential oil in the 90's, but were at that time not found in beer[85]. It concerns 2 allylic alcohols (caryophylladienol= caryophylla-4(12),8(13)-diene-5-ol and caryophyllenol= caryophylla-3,8(13)-diene-5-ol), a caryophyllene derived aldehyde (6(5→4)-abeo-caryophyll-8(13)-en-5-al), and a ketone (dihydrocaryophyllene-5-one). The ketone as well as the 2 allylic alcohols each have 2 isomers[189]. According to Tkachev[189], the ketone that was detected in hop oil by Yang and coworkers[85] is the S isomer, whereas the detected caryophylladienol and caryophyllenol structure are the α isomer.

We identified caryophylla-4(12),8(13)-diene-5-ol (n°38) in beer B. However, using GC-MS, no distinction between caryophylla-4(12),8(13)-diene-5α-ol and caryophylla-4(12),8(13)-diene-5β-ol can be made, due to the identical retention index and mass spectrum. Therefore, the isomeric form of compound n° 38 is not further specified in **Table 3-7**. Dresel and coworkers assigned the general name, *i.e.* 'caryophylladienol', to this volatile after detecting it in wort samples, hopped with cv. Amarillo, and in both a kettle and dry hopped beer, brewed with cv. Wilamette[197]. Although this caryophyllene derivative is only a minor compound, it can easily be recognised by the distinct mass fragment at m/z 136 in the mass spectrum.

Caryophylla-3,8(13)-diene-5α-ol and caryophylla-3,8(13)-diene-5β-ol can, despite their identical mass spectrum, be distinguished on the basis of their retention index[189]. The β-isomer was detected quite recently in hop oil and in ale beer[146], whereas the α-isomer was detected in hop oil several years ago[85]. Our research group recently detected the latter compound in both a kettle and dry hopped beer, brewed with a single hop variety cv. Wilamette[197]. Also Tressl and coworkers reported about the presence of 'caryophyllenol' in beer, although the isomeric form was not further specified[88]. In this work, we detected both isomers in beer A and B (n° 45 and 54, **Table 3-7**).

In contrast to the caryophyllene allylic alcohols discussed above, the caryophyllene derived aldehyde (compound n°21 in **Table 3-7)** has hitherto never been detected in beer. This could possibly be attributed to confusion with caryophyllene oxide, which has a similar retention index and depicts a highly similar mass spectrum (see **Figure 3-7**). Nevertheless, these two compounds can be distinguished since the mass spectrum of 6(5→4)-abeo-caryophyll-8(13)-en-5-al shows high relative intensities for the fragment ions at m/z 41, 107 and 164, whilst the caryophyllene oxide mass spectrum is characterised by higher intensities at m/z 43, 109 and 161. Based on its mass spectrum and retention index, we determined compound n° 21, detected in beer B, as 6(5→4)-abeo-caryophyll-8(13)-en-5-al.

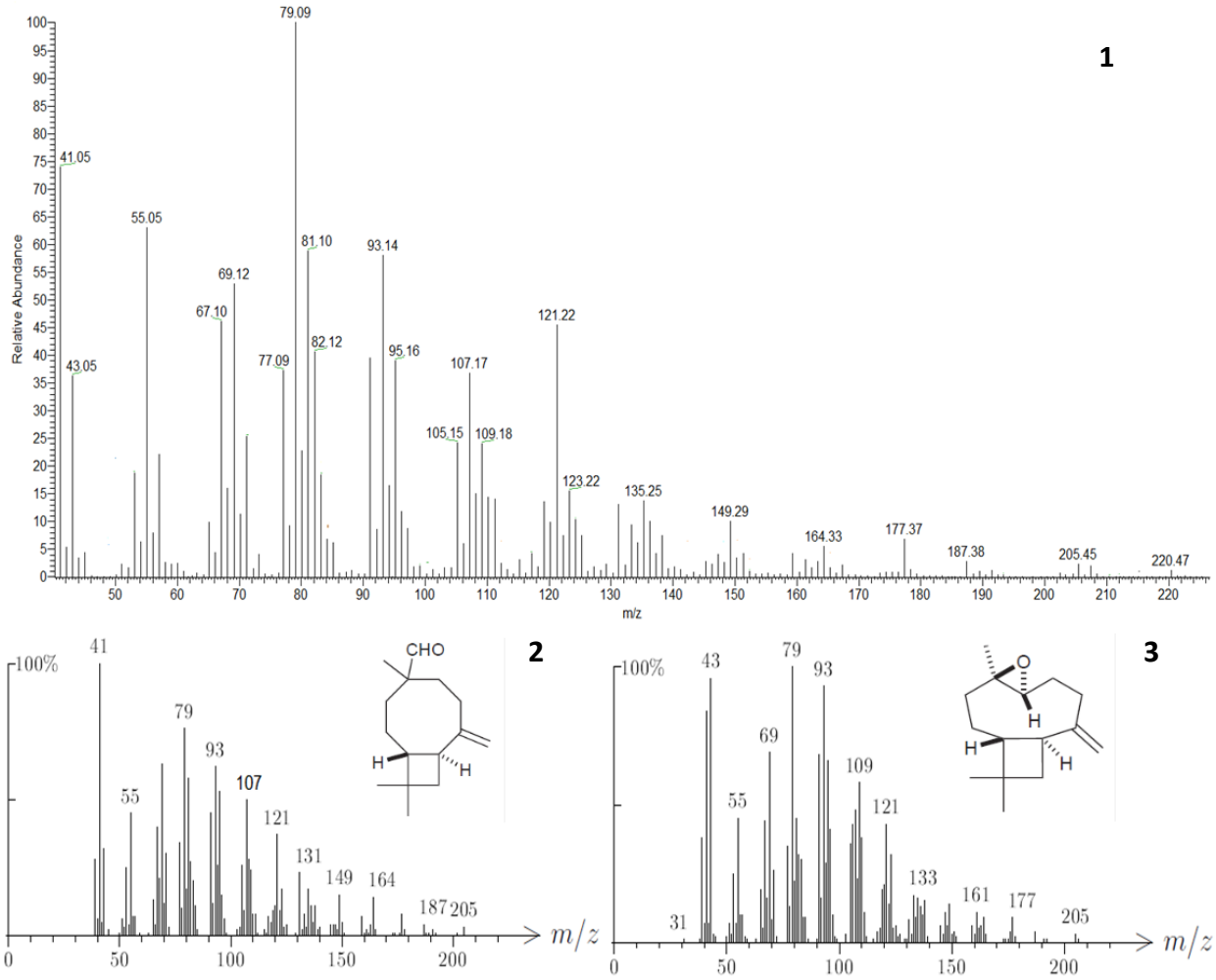


Figure 3-7. Mass spectra of caryophyllene oxide and a caryophyllene-derived aldehyde. 1. Mass spectrum of compound n° 21 (in accordance with **Table 3-7**), detected in beer B. **2.** Mass spectrum of 6(5→4)-abeo-caryophyll-8(13)-en-5-al[189]. **3.** Mass spectrum of caryophyllene oxide[189].

The caryophyllene derived ketone (n° 14) is also detected in this study for the first time in beers A and B. Both the S and R isomer depict highly similar mass spectra[189]. However, on the basis of the calculated Retention index the compound was identified as 4S-dihydrocaryophyllene-5-one, which was already reported in hop essential oil[85]. The experimental mass spectrum of this compound is depicted in **Figure 3-8**.

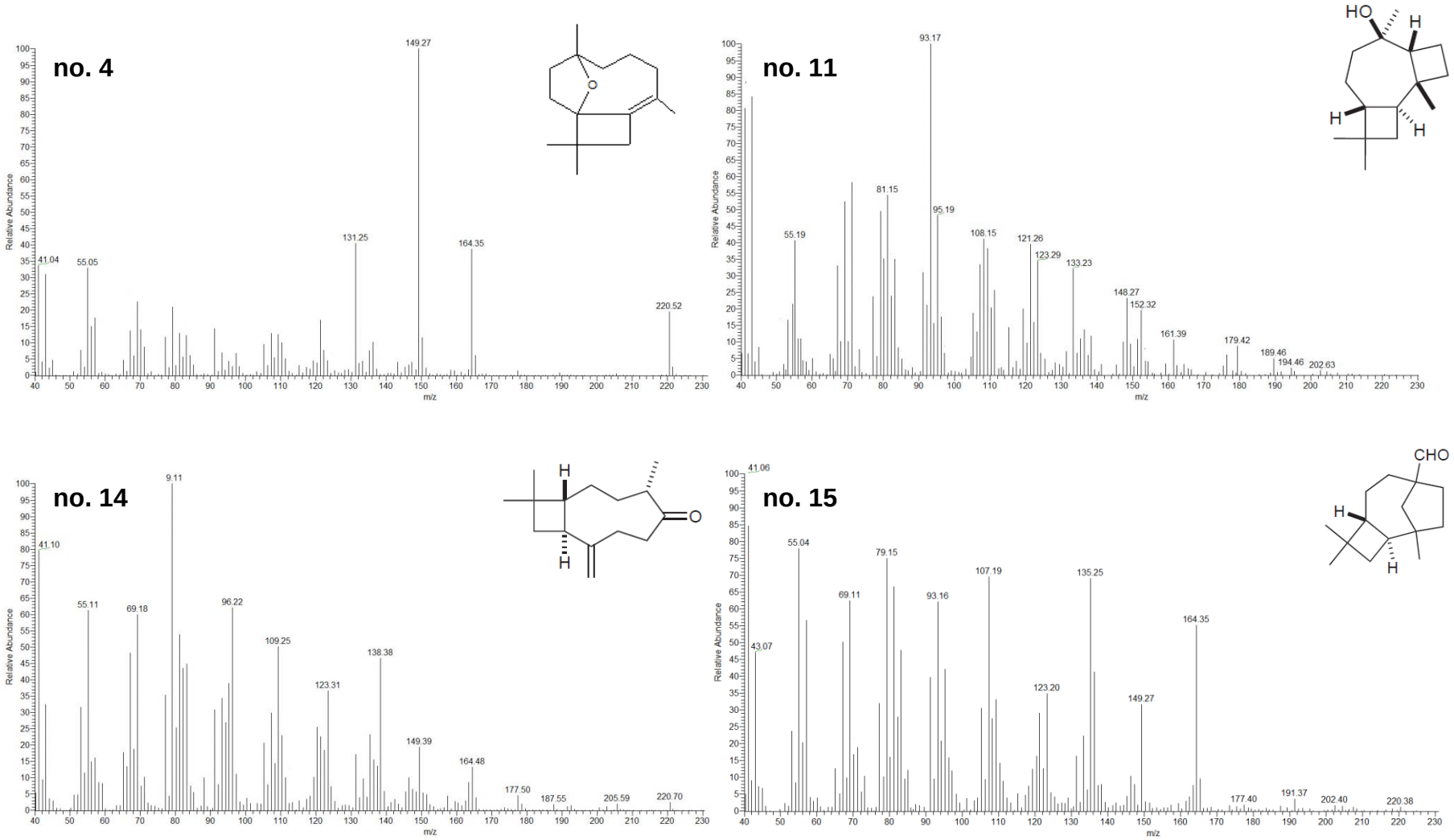


Figure 3-8. Experimental mass spectra of compound no. 4 (1,5,8,8,-tetramethyl-12-oxa-5-tricyclo[7.2.1.0^{6,9}]dodecene), no. 11 (iso-korajol = (1R,2R,5S,6R,9R)-2,6,10,10-tetramethyltricyclo[7.2.0.0^{2,5}]undecan-6-ol), no. 14 (4S-dihydrocaryophyllene-5-one) and no. 15 6(5→4)abeo-8,12-cyclo-caryophyllan-5-al. Chemical structures are reproduced from literature data[138,189].

In conclusion, both the abovementioned caryophyllene derived aldehyde and ketone were reported in hop oil but have not been detected in beer. Next to these compounds, we would like to report on 2 other compounds, which have hitherto neither been detected in hops, nor in beer. It concerns compound n° 15, which was identified as 6(5→4)-abeo-8,12-cyclo-caryophyllan-5-al, and compound n° 11, identified as iso-korajol on the basis of both retention index and mass spectrum[189]. The experimental mass spectra and chemical structures of these compounds are depicted in **Figure 3-8**.

We also wish to underline the presence of a series of compounds which have not frequently been reported in literature data. One of them is compound no. 4, which was already identified in a mixture of reaction products, obtained via reflux boiling of humulene epoxide II and III by Yang and Deinzer[138] and was later detected in beer[84]. We were able to tentatively identify compound n° 4 as 1,5,8,8,-tetramethyl-12-oxa-5-tricyclo[7.2.1.$0^{6,9}$]-dodecene on the basis of its highly characteristic mass spectrum. According to Yang and Deinzer, the mass spectrum is characterised by high fragment ion intensities at m/z 55, 131, 149 and 164 (relative intensities of 23%, 31%, 100% and 71 %, respectively) and shows an intense molecular ion at m/z 220 (relative intensity of 45%)[138], which is exactly identical to our experimental mass spectrum for compound no. 4 (see **Figure 3-8**).
Clovenol (n° 22) and junenol (n° 31) were detected in beer in this study. The latter compound was mentioned in the context of beer by Tressl and coworkers[88], although they did not find this compound in hops[82]. Finally, 2 humulene derived oxygenated sesquiterpenoids, containing an allylic alcohol in their chemical structure (n° 29, 56) were detected. Humulenol II also belongs to the chemical group of humulene-derived allylic alcohols. Next to humulenol II, Yang and Deinzer also detected another humulene allylic alcohol, *i.e.* 2,6,6,9-tetramethyl-2,4,8-cyclo-undecatrien-1-ol, in the mixture of hydrolysates of humulene epoxide II and III and also in hop oil and beer[84,138]. This compound might match our compound n° 29 or 56, although this can not be verified since the authors did not provide spectral information.

From **Table 3-7**, it is clear that the hop-derived OS spectrum strongly varies among the beers. Some compounds are found in all the investigated beers and show a relatively high peak area (*e.g.* humuladienone, humulene epoxide I, II, III, humulol, humulenol II, τ-cadinol and humulene diepoxide A). Strikingly, these compounds were also thoroughly investigated in literature. Other compounds are not detected in all beers or only show minor peaks, which might explain why they were frequently overlooked in earlier studies. Indeed, several years ago Siebert stated: "It is highly likely that, despite the advances in analytical methodology, we still can't measure compounds at low enough concentrations to know all

those responsible for hoppy flavour"[113]. This statement seems valid up to date, since new minor constituents are still found flavour-active[114,124,146]. Certainly worth underlining is the fact that several OSs, detected in beer-derived SPE fractions (**Table 3-7**, n° 4, 14, 15, 17, and 29), were previously indicated as compounds 'newly' formed upon boiling of total hop essential oil (cv. Saaz) (**Chapter 2**). This observation provides strong indications for *de novo* formation of OSs during real wort boiling.

3.3.6 GC-O analysis for characterisation of flavour-active zones in SPE-derived fractions of commercial kettle hopped lager beer

To obtain insights in which particular sesquiterpenoids may contribute to the (kettle) hoppy aroma of beer, sensory assessment was performed via GC-O analysis on the SPE fractions derived from beer B. Beer B was chosen for profound olfactometric evaluation on the basis of its relatively high levels of OSs in the SPE fractions, as reported earlier (see **Figure 3-1**).

Table 3-8. Compounds detected in flavour-active chromatographic regions in the aromagrams, obtained via GC-O analysis of the SPE-derived 70% and 80% ethanol fractions of beer B. All identifications are based on a match for both RI and mass spectrum. RI= calculated retention index, start= start flavour-active region, end= end flavour-active region. DF= detection frequency. Compounds in bold: DF ≥ 3.

RI start	RI end	Compound	70% DF out of 3	80% DF out of 3	total DF out of 6
1463	1468	1,5,8,8,-Tetramethyl-12-oxa-5-tricyclo[7.2.1.0^{6,9}]dodecene	2	0	2
1468	1472	Unclear mass spectrum	2	0	2
1489	1494	3,5-Di-tertylbutyl-phenol/ δ-cadinene	2	0	2
1502	1504	Iso-korajol	1	1	2
1517	1518	Unclear mass spectrum	1	1	2
1520	1521	α-Calacorene	2	0	2
1522	**1524**	**Unclear mass spectrum**	**3**	**1**	**4**
1536	1541	Unknown (m/z 93, 205, 220)	2	0	2
1537	**1539**	**β-Calacorene**	**2**	**1**	**3**
1539	1542	Unclear mass spectrum	0	2	2
1548	1549	Caryolan-1-ol/humuladienone	1	1	2
1557	1559	Clovenol	2	0	2
1569	1574	Unclear mass spectrum	0	2	2
1569	**1572**	**Humulene epoxide I**	**2**	**3**	**5**
1573	**1576**	**Humulol**	**2**	**2**	**4**
1584	**1586**	**Unclear mass spectrum**	**2**	**2**	**4**
1587	1590	Unknown (m/z 81, 123, 135, 161, 179, 189, 204, 207)/ humulene allylic alcohol	1	1	2
1594	1600	Junenol	1	1	2
1596	1598	Unclear mass spectrum	2	0	2
1601	1604	Humulene epoxide III	1	1	2
1605	**1608**	**Humulenol II**	**2**	**2**	**4**
1609	**1613**	**Caryophylla-4(12),8(13)-diene-5-ol/ phenyl ethyl hexanoate**	**2**	**3**	**5**
1616	**1619**	**τ-Muurolol**	**2**	**2**	**4**
1616	1619	Cubenol	1	1	2
1622	1625	β-Eudesmol	1	1	2
1628	1629	α-Cadinol	2	0	2
1627	1630	3Z-Caryophylla-3,8(13)diene-5α-ol	1	1	2
1635	**1639**	**14-Hydroxy-caryophyllene**	**1**	**2**	**3**
1636	1641	Unknown (m/z 93, 137)	0	2	2
1643	**1645**	**Cadalene/ 3Z-caryophylla-3,8(13)diene-5β-ol**	**2**	**3**	**5**
1668	**1671**	**Unclear mass spectrum**	**1**	**2**	**3**

Table 3-8 displays odour-active regions (31 in total) that were detected at least twice upon GC-O analysis and, where possible, the identity of the compounds detected in the respective odour-active regions is reported.

A relatively high number of chromatographic regions showed weak odour-activity based on the detection frequency (DF=2), and, in several cases, it was impossible to estimate the identity of the compound responsible for the perceived odour (due to low quality mass spectra or low level of the compound below the detection limit). However, we were able to (tentatively) identify 1,5,8,8,-tetramethyl-12-oxa-5-tricyclo[7.2.1.0^{6,9}]dodecene (literature odour descriptors: 'cedar', 'camphor'[84,138]), δ-cadinene (literature odour descriptor: 'herbal'), iso-korajol, clovenol, junenol, and α-calacorene (literature odour descriptor: 'wood'[124,183]) in weak odourous zones. The latter compound was already detected in an odour-active region of a hop oil-derived OS fraction[124] and has been related to the citrusy and spicy character of beer[183]. In this study, its isomer β-calacorene was also tentatively identified in the odourous zone ranging from RI 1537-1539.

In this study, caryolan-1-ol and humuladienone were detected in the odour-active region from RI 1548-1549. Shimazu and coworkers[16] already proposed humuladienone as a flavour-impact compound for hoppy aroma, since its content in beer (30 – 70 ppb) is near to the flavour threshold (100 ppb). Several years ago, humuladienone was detected in beer via GC-O[11] and, only recently, Van Opstaele and coworkers[124] reported on a highly flavour-active zone in a spicy hop essence that consists of humuladienone and caryolan-1-ol. The presence of caryolan-1-ol in beer has been demonstrated several times[18,88,160,197], but the question whether caryolan-1-ol is odour-active remains unanswered.

Humulene epoxide III, described in literature data as 'cedar'[84], was present in the odour-active zone ranging from RI 1601 – 1604. Although this compound is a humulene oxidation product, its chromatographic peak was yet relatively small, probably due to conversion to various hydrolysis and isomerisation products[138].

Bicyclic sesquiterpenoid alcohols (*e.g.* cubenol, β-eudesmol, α-cadinol) were detected in specific odour-active regions. In literature, β-eudesmol (RI 1622 – 1625) has been proposed as a key aroma compound related to hoppy characteristics in beer using PCA by Inui and coworkers[183].

The odourous zone comprising humulene epoxide I showed high flavour-activity (DF=5). It has been suggested in literature that humulene epoxide I contributes to hop aroma in beer since its concentration in beer (*e.g.* 125 ppb[88]) may be far above its reported threshold value (10 ppb in water[18]). A relatively high peak area of humulene epoxide I in the registered chromatogram in this study, in particular in comparison to the peak areas of humulene epoxide II and III, points to high levels in beer. In general, the concentration of humulene

epoxide I in hops is lower than the level of humulene epoxide II[82]. However, the reverse is observed in beer, which can be assigned to the fact that humulene epoxide I is fairly resistant to hydrolysis whereas humulene epoxide II and III can be extensively converted into a series of alcohols[28,138]. Humulene epoxide II was not found to be odour-active on the basis of our sniffing analyses and although this compound was previously detected in an odour-active region of an OS hop oil fraction, the odour was proposed to be imparted by minor co-eluting constituents[124].

In contrast to humulene epoxide II, we observed relatively high levels of humulenol II in a highly flavour-active region (RI 1605-1608, DF=4) in the registered aromagrams. The higher level of humulenol II compared to the level of humulene epoxide II is possibly due to the chemical conversion of humulene epoxide II to humulenol II as was reported in literature[10,17,18,138]. Furthermore, our findings on the odour-activity of humulenol II are in agreement with literature data. Peacock and coworkers[18] found that humulenol II should at least be partly responsible for hoppy aroma of beer, in particular for beers brewed with traditional aroma hop varieties (noble kettle hop aroma)[17,18], whereas Lam and coworkers reported on the contribution of humulenol II to the herbal/spicy note of beer[10].

Finally, our GC-O results clearly show the presence of particular sesquiterpene alcohols, *i.e.* humulol, τ-muurolol, caryophylla-4(12),8(13)-diene-5-ol, 14-hydroxy-caryophyllene and 3Z-caryophylla-3,8(13)diene-5β-ol, in several highly odour-active regions. Most of these constituents have already been reported in earlier studies as potential flavour-impact compounds for hop(py) aroma. For example, 14-hydroxy-caryophyllene was found to be flavour-active by Eyres and coworkers[114] on the basis of GCxGC-TOFMS and GC-O analysis of the spicy fraction of hops, while 3Z-caryophylla-3,8(13)diene-5β-ol has been indicated as a character-impact compound for the hop aromatic character of ale beer[146]. Although the contribution of humulol to hoppy aroma of beer has been questioned because of its high threshold value[83], we were able to find an indication for the odour-activity of humulol in the investigated beer. To our knowledge, caryophylla-4(12),8(13)-diene-5-ol is reported for the first time as potential odour-impact compound in beer.

In conclusion, GC-O clearly demonstrated the odour-activity of OS fractions isolated from a commercial kettle hopped lager beer. Moreover, many of the compounds identified in flavour-active zones were also indicated upon GC-O analyses of the hop oil-derived SPE fractions (see **section 3.3.4**). It concerns 1,5,8,8,-tetramethyl-12-oxa-5-tricyclo[7.2.1.$0^{6,9}$]dodecene , an unknown oxygenated sesquiterpenoid (m/z 93, 205, 220), clovenol, humulene epoxide I, a humulene allylic alcohol, humulene epoxide III, humulenol II, caryophylla-4(12),8(13)-diene-5-ol, cubenol, (3Z)-caryophylla-3,8(13)-diene-5α-ol, and

(3Z)-caryophylla-3,8(13)-diene-5β-ol. Also an unknown compound at RI 1628 (co-eluting with compound characterised by m/z at 79, 80, 81, 164, 222) (see **Table 3-6**) proved highly odour-active upon GC-O of the hop oil-derived fractions. This compound might coincide with 14-hydroxy-β-caryophyllene (RI 1635) (see **Table 3-8**), detected in an odour-active region of the beer-derived SPE fractions. Further investigation concerning this compound is however mandatory.

Two decades ago, in the context of flavour-active compounds and the deficiency of analytical tools to detect them at that time, Siebert stated[113]: "Two approaches appear appropriate in this situation. The more tedious is to fractionate a hoppy beer, at each step adding each fraction back to beer for tasting… An alternative approach is to use the separating power of gas chromatography combined with the sensitivity of the human nose as detector". Summarised, two research approaches are suggested, *i.e.* reconstitution/omission experiments and GC-olfactometry. Although in this study we did not add each fraction back to beer, we basically combined the two proposed approaches by fractionating beer and performing GC-O on these fractions. This approach allowed us to point out potential flavour-active OSs. Most of them were already related to hoppy aroma of beer decades ago, although GC-olfactometry was not available at that moment. Later on, the role of the α-humulene and β-caryophyllene oxidation and hydrolysis products in particular was seriously questioned since at first they were not detected via GC-O. However, more recently, several of these products were found to express green, woody, cedar and spicy odours[114,124,146] and also our current work suggests flavour-activity of OSs. With respect to the still ongoing debate about flavour-active volatiles imparting the illusive kettle hoppy aroma, (non-identified) OSs once again arise as potential aroma impact compounds.

3.4 Conclusions

A relatively simple yet effective Solid Phase Extraction (SPE) based methodology was developed and combined with HS-SPME-GC-MS in order to improve separation, detection and identification of hop oil-derived volatiles present in unboiled and boiled hop essential oil (cv. Saaz) on the one hand, and, on the other hand, OSs in commercial kettle hopped lager beers. This methodology allowed for a detailed fingerprinting of the hop oil-derived volatile profile and proved to be an interesting tool to overcome the problem of co-elution. Moreover, the application of this methodology for characterisation of fractions derived from boiled hop oil and hop oil-derived volatiles in beer has not been performed before. Application of this method to beer in particular resulted in a profound characterisation of OSs in beer and allowed for demonstration of the presence of iso-korajol, 4S-dihydrocaryophyllene-5-one, 6(5→4)-abeo-8,12-cyclo-caryophyllan-5-al and 6(5→4)-abeo-caryophyll-8(13)-en-5-al in lager beer for the first time. By comparing unboiled and boiled hop oil fractions, a higher number of compounds as detected in **Chapter 2** were found to be characteristic for boiled hop oil and thus formed *de novo* upon boiling. The presence of these compounds indicates chemical oxidation of SHCs during boiling and, since several of these compounds were detected in the commercial kettle hopped lager beers, also oxidation of SHCs during real brewing practice. Moreover, a large series of compounds that previously showed an increase in their level upon boiling (see **Chapter 2** and **section 3.3.3**) were detected in these lagers.

The hop-oil derived SPE fractions were spiked to non-aromatised iso-α-acid bittered lager beer for sensory evaluation. The fractions B70 and B80, containing high levels of 'spicy' compounds and OSs in particular, showed interesting flavour characteristics with respect to 'kettle hop' aroma. Therefore, GC-O was performed to search for individual odour-active compounds. α-Humulene-derived epoxides and both α-humulene and β-caryophyllene derived alcohols were frequently detected in these flavour-active zones. Many of these compounds were also found to elute in flavour-active intervals upon GC-O analysis of the beer-derived SPE fractions, implying that these compounds may be important regarding the hop aroma characteristics of kettle hopped lager beers, in agreement with the general view.

The innovative aspect of this chapter comprises the combination of two completely different approaches, *i.e.* fractionation of boiled hop oil on the one hand and hop oil-derived volatiles from commercial kettle hopped lagers on the other hand, and, performing GC-O on these fractions to indicate common compound classes that are potentially relevant to kettle hop aroma. Our observations strongly suggest that *de novo* formation of OSs also occurs during real brewing practice and, consequently, that the performed lab scale boiling experiments are relevant.

Chapter 4

FLAVOUR-ACTIVITY OF SESQUITERPENE OXIDATION PRODUCTS, FORMED UPON LAB SCALE BOILING OF A HOP ESSENTIAL OIL-DERIVED SESQUITERPENE HYDROCARBON FRACTION (CV. SAAZ)

Chapter 4 corresponds to:

Praet,T.; Van Opstaele, F.; Steenackers, B.; De Vos, D.; Aerts, G. and De Cooman, L. Flavour-activity of sesquiterpene oxidation products, formed upon lab scale boiling of a hop essential oil-derived sesquiterpene hydrocarbon fraction.

J. Am. Soc. Brew. Chem., **2016** (accepted for publication)

Chemical-analytical profiling of hop oil-derived sesquiterpene hydrocarbon fraction (cv. Saaz). Boiling of sesquiterpene hydrocarbon fraction and SPE isolation of formed sesquiterpene oxidation products. Sensory evaluation of sesquiterpene oxidation products via GC-O and evaluation by taste panel upon addition of oxidation product fraction to non-aromatised iso-α-acid bittered lager beer.

Tatiana Praet and Nedelina Nikolova (master student) performed the experiments. The final manuscript was written by Tatiana Praet and revised and adapted after critical input by Prof. Luc De Cooman and Dr. Filip Van Opstaele.

4 FLAVOUR-ACTIVITY OF SESQUITERPENE OXIDATION PRODUCTS, FORMED UPON LAB SCALE BOILING OF A HOP ESSENTIAL OIL-DERIVED SESQUITERPENE HYDROCARBON FRACTION (CV. SAAZ)

4.1 Introduction

Despite the increasing number of craft brewers supplying numerous ales with pronounced and characteristic hop-derived fruity flavours, lager beer still has a prevailing market share in worldwide beer consumption. Brewing beers with a refined 'hoppy' aroma is therefore high on the priority list for brewers of traditional European lagers[158]. Consequently, the aroma of beer has been studied thoroughly for several decades and important work has been conducted to identify the compounds responsible for flavour-activity[11,26,166,176].

Oxygenated sesquiterpenoids (OSs) have been proposed to be related to a 'spicy' aroma impression[19], which is in its turn associated with noble or kettle hop aroma[27]. Various humulene and caryophyllene oxidation products and their hydrolysis products were first proposed as important contributors[10,17,84,85,138,174]. Lately, several caryophyllene derivatives were found to be flavour-active[114,124,146] and many researchers claimed that here had to be yet unidentified OSs with high flavouring potential[12,19,114,139,146]. In **Chapter 3**, humulene epoxide I, humulol, humulenol II, caryophylla-4(12),8(13)-diene-5-ol, τ-muurolol, 14-hydroxy-β-caryophyllene and 3Z-caryophylla-3,8(13)diene-5β-ol were found in flavour-active zones upon GC-O analysis of spicy fractions, derived from a commercial lager beer. Taken together, it has been shown that particular α-humulene and β-caryophyllene derivatives show flavouring potential. However, although there is clearly a correlation between these compounds and 'hoppy aroma', a cause-effect relationship has not been proven.

Despite the decades of research that have preceded our current knowledge concerning 'hoppy' aroma, new scientific insights are only painstakingly achieved. In spite of the availability of novel extraction and analytical methods for volatiles (such as headspace solid phase microextraction (HS-SPME)[6,123,124,146,172,197,198,201–203], solvent assisted flavour evaporation (SAFE)[204] and multidimensional separation techniques[114,124,183] and recent developments such as the electronic nose (E-nose)[205–207], until today the human nose remains the most sensitive instrument to detect flavour-activity of individual compounds[208,209]. However, OSs express rather subtle odours and the 'hoppy' flavour impression is probably the result of additive, synergetic and/or antagonistic effects between different compounds[14,113]. Comparison of non-hopped beers with hopped beers to detect

hop-derived compounds contributing to 'hoppy' flavour, did not prove very successful, and to gain more fundamental insights, several researchers performed boiling experiments with humulene and caryophyllene (derivatives) or hop oil fractions in simplified model solutions[17,27,28,85,135,138,139], revealing the formation of various oxygenated derivatives and hydrolysis products and changes in the flavour attributes. Such a fundamental approach, in combination with improved analytical tools, should allow for obtaining new insights into formation of OSs and their role in 'kettle hop' aroma. In **Chapter 2**, total hop essential oil cv. Saaz was boiled on a lab scale, revealing changes in the hop-derived volatile profile upon boiling. A general increase in the level of spicy compounds could be observed, which was attributed to increases in the levels of OSs (*e.g.* humuladienone, caryophyllene oxide, humulene epoxide I-III, caryophylla-4(12),8(13)-diene-5α/β-ol, (3Z)-caryophylla-3,8(13)-diene-5α/β-ol, humulol), and qualitative changes (*i.e.* newly formed compounds upon boiling) were pinpointed (*e.g.* 4-S-dihydrocaryophyllene-5-one, 1,5,8,8-tetramethyl-12-oxa-5-tricyclo[7.2.1.$0^{6,9}$]dodecene). Interestingly, addition of boiled hop essential oil (cv. Saaz) to iso-α-acid-bittered lager beer reduced 'malty/worty' flavours and increased 'spicy' and 'hoppy' notes. In this chapter, we focus on sesquiterpene oxidation products (SOPs) and their link with 'hoppy' aroma. To this end, we performed lab-scale boiling of a varietal sesquiterpene hydrocarbon (SHC) fraction and aimed at enrichment of the oxidation products and characterisation of this enriched fraction both analytically ((tentative) identification of OSs via HS-SPME-GC-MS (HS-SPME gas chromatography-mass spectrometry) and from a sensory point of view (via sensory evaluation by taste panels and GC-O).

4.2 Experimental

4.2.1 Chemicals

The following reference compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical grade: (-)-caryophyllene oxide (≥98.5%); (-)-clovene (≥90%, sum of enantiomers); (-)-*trans*-caryophyllene (≥98.5%); (-)-β-pinene (≥99.0%); (+)-aromadendrene (≥97.0%, sum of enantiomers); (+)-α-pinene (≥98.5%); (R)-(+)-limonene (97%); 2-heptanol (98%); 2-tridecanone (≥97.0%); *trans*-β-farnesene (≥90%); α-humulene (≥96%); β-myrcene (≥95.0%) α-cubebene (97%) and α-copaene (≥90%, sum of enantiomers). Iso-caryophyllene and oxygenated sesquiterpenoid (OS) mixtures of reference compounds were prepared as described in **section 2.2.1.2**.

4.2.2 Plant material

See **section 2.2.2**.

4.2.3 Isolation of hop essential oil from hop pellets

Hop oil was extracted from hop pellets cv. Saaz (T90) via the steam distillation method described in **section 2.2.3.1**. The hop essential oil was collected and diluted in EtOH (1/100 v/v hop essential oil/HPLC grade EtOH). Diluted oil was poured into dark brown screw-capped glass vials (Chromacol, amber glass, 20 mL, Welwyn Garden City, UK) and stored in the freezer (-18°C) until further analysis.

4.2.4 Enrichment of SHCs from hop essential oil via SPE

SPE was used to fractionate the steam distilled hop essential oil. Varian Bond Elut C18 (octadecyl silica) cartridges (500 mg, 6 mL, Agilent Technologies, Lake Forest, USA) were pre-conditioned with 10 mL of MQ-water (purified water), 10 mL of EtOH (≥ 99.8%, VWR International, Zaventem, Belgium), and finally 10 mL EtOH/water (1/1; v/v EtOH/MQ-water). The sample (hop oil diluted in EtOH) was further diluted with MQ-water (to make a 1/1 v/v EtOH/MQ-water solution), whereupon 6 mL was pipetted on the C18 column and eluted. Next, hop oil compounds adsorbed to the C18 stationary phase were fractionated by gradually increasing the EtOH concentration of the eluent (3 mL) from 50% EtOH/MQ-water (v/v) to 100% EtOH and eluting a final time with 100% EtOH to obtain full desorption of hop oil compounds. Each fraction was collected separately, brought into screw-capped brown glass vials (20 mL) and stored in the freezer (-18°C) until further analysis. All the fractions were diluted in water (100 µL fraction, 4.5 mL MQ-water, 100 µL 2-heptanol (internal standard) stock solution (253 mg/L)) in HS-SPME vials (Chromacol, clear glass, 20 mL, Welwyn Garden City, UK) and closed with bimetal magnetic crimp caps containing a

silicone/Teflon septum (Interscience, Louvain-la-Neuve, Belgium), prior to HS-SPME-GC-MS analysis (split ratio 1:10) (triplicate analysis).

Hop oil compounds were classified into monoterpene hydrocarbons, floral compounds (oxygenated monoterpenoids, aliphatic and branched esters, alcohols, ketones, aldehydes etc.), SHCs and spicy compounds (mainly OSs and aliphatic and branched esters, alcohols, ketones and aldehydes), named after the odour that is perceived when isolating these fractions from hop essential oil via SPE[184]. The normalised peak areas (peak area normalised against 2-heptanol as internal standard to compensate for HS-SPME variation) of each compound class in each fraction are depicted in **Figure 4-1**, which shows that the fraction eluting with 90% EtOH contains the highest level of SHCs. The SHC concentration in this fraction was determined semi-quantitatively via GC-FID[175] (triplicate analysis). The concentration was determined at 2.29 ± 0.08 g/L, and the estimated purity (on the basis of relative areas of SHCs in the GC-FID chromatogram) was 95.95 ± 0.07%. In conclusion, the 90% EtOH fraction contains the highest SHC level in combination with a high purity; consequently, this fraction was further indicated as the sesquiterpene hydrocarbon (SHC) fraction.

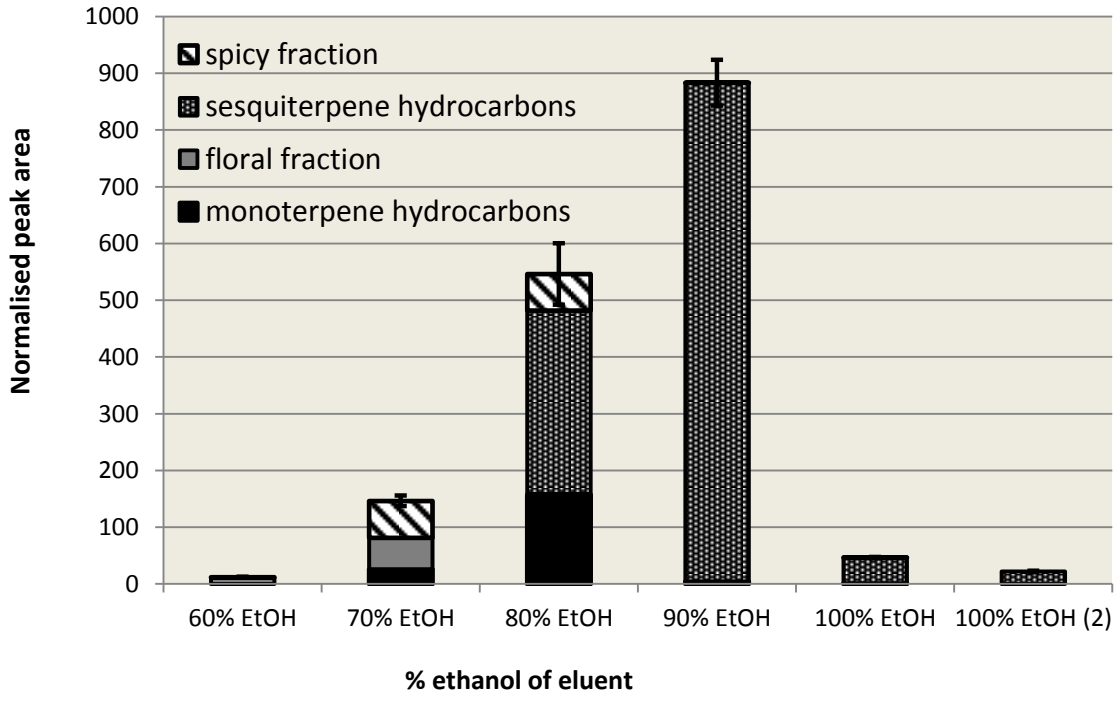


Figure 4-1. Normalised peak area (normalised for peak area of 2-heptanol as an internal standard) of monoterpene hydrocarbons, floral compounds, sesquiterpene hydrocarbons and spicy compounds, detected in HS-SPME-GC-MS chromatograms of hop oil-derived fractions, obtained via SPE. Standard deviation on basis of total peak area (n=3).

4.2.5 Boiling of SHC fraction

Twelve closed HS-SPME vials, containing SHC fraction (500 mg/L, dilution in MQ water, total volume of 5 mL), were prepared, of which 2 samples remained unboiled as a reference and 10 vials were boiled (1, 2, 3, 4 and 5 h, in duplicate) in the incubation oven of the CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland); the oven temperature was 100°C, and the stirring of samples was 250 rpm with 5 s on and 2 s off. After boiling, the vials were removed and cooled in the cooler (3°C) of the CombiPAL autosampler. Before HS-SPME-GC-MS analysis (split ratio 1:50), 100 µL internal standard (12.157 g/L 2-heptanol) was added with a syringe (100 µL, Hamilton, Reno, USA) through the septum.

4.2.6 Isolation of OSs from boiled SHC fraction by SPE

HS-SPME vials containing SHC fraction in MQ water (1000 mg/L) were boiled for 2 h, cooled, and finally diluted with an equal volume of EtOH. SPE fractionation was performed as described above, and the resulting fractions were analysed by HS-SPME-GC-MS. The normalised peak areas are depicted in **Figure 4-2**. The fraction eluting with 70% EtOH contained the highest level of spicy compounds (predominantly OSs): 7.6% monoterpene hydrocarbons, 0.3% floral compounds, 0.0% SHCs and 92.2% spicy compounds (based on relative areas). On the basis of GC-FID analysis, the OS concentration in the 70% EtOH fraction was determined at 0.41 ± 0.09 g/L (n=3). This particular fraction was further indicated as the sesquiterpene oxidation product (SOP) fraction and was characterised via HS-SPME-GC-MS (splitless injection, triplicate analysis).

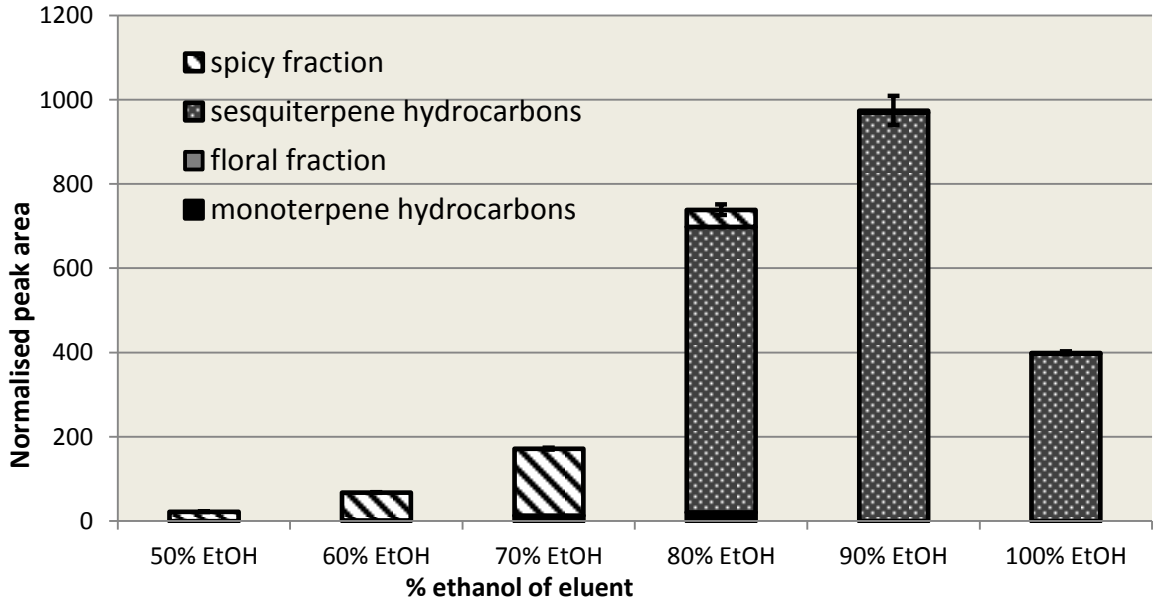


Figure 4-2. Normalised peak area (normalised for peak area of 2-heptanol as an internal standard) of monoterpene hydrocarbons, floral compounds, sesquiterpene hydrocarbons and spicy compounds, detected in HS-SPME-GC-MS chromatograms of SPE fractions of boiled hop sesquiterpene hydrocarbons (SHC) (boiling time: 2 h). Standard deviation on basis of total peak area (n=3).

4.2.7 HS-SPME-GC-MS

Hop-derived volatiles were extracted via headspace solid-phase microextraction (HS-SPME) (fibre coating: polydimethylsiloxane (PDMS), extraction time: 45 min, extraction temperature: 60°C) as previously described in **section 2.2.6**. Both splitless and split (ratio 1:10 and 1:50) injections were performed. GC conditions for separation of the volatiles were described in **section 2.2.6**. In this study, two different oven programs were used for separation of the volatiles via the RTX-1 capillary column (nonpolar fused silica column, dimensions: 40 m x 0.18 mm x 0.25 µm): (1) 1 min at 40°C, ramp of 10°C/min, hold 1 min at 72°C, ramp of 2°C/min, hold 1 min at 137°C, ramp of 1°C/min, hold 1 min at 160°C, ramp of 10°C/min, hold 3 min at 250°C. (2) 1 min at 40°C, hold for 3 min, ramp of 1°C/min, hold 1 min at 187°C, ramp of 20°C/min, hold 5 min at 250°C. The first oven program was used for routine analysis. The second program permited maximal separation of all extracted volatiles and was used for identification of SHCs in the SHC fraction. Mass spectrometric detection of volatiles was performed as described in **section 2.2.6**.

4.2.8 GC-O

Flavour-active constituents in the SOP fraction were determined via GC-olfactometry as described by Van Opstaele and coworkers[123]. Olfactory global analysis (OGA) was applied on the SOP fraction (splitless injection, 10-fold dilution of SOP fraction in MQ-water, 5 assessors, analyses in duplicate) to determine the flavour-activity of the compounds. Assessors were thoroughly trained for odour detection and description of OSs using total hop essential oils (both boiled and unboiled), spicy fractions (prepared as described by Van Opstaele and coworkers[124]) and mixtures of OSs that were obtained via chemical treatment of α-humulene and β-caryophyllene (**section 2.2.1.2**). The detection frequency (DF) represents how many times the odourant was detected out of the 10 analyses.

For investigation of the flavour-activity of caryophyllene derived allylic alcohols, GC-O was performed on the β-caryophyllene allylic alcohol mixture (split (ratio1:10) injection, 40 mg/L in MQ water) as well as on disrupted birch buds (containing *Betula* essential oil, rich in 14-hydroxy-β-caryophyllene) (splitless analysis, 250 mg). Analyses on these mixtures, containing high levels of particular caryophyllene allylic alcohols which do not co-elute with other volatiles under the applied GC conditions, permit unambiguous allocation of a perceived odour to a particular caryophyllene-derived alcohol. Three assessors showing sensitivity for caryophyllene allylic alcohols were selected to perform these analyses in triplicate.

4.2.9 Sensory analysis of the SOP fraction in non-aromatised iso-α-acid-bittered beer

Odour and aroma characteristics of the SOP fraction were evaluated via descriptive sensory analysis by our trained taste panel (12 panellists, training performed by using reference compounds, *e.g.* linalool, β-myrcene, nonanal, 2-undecanone, α-humulene, β-caryophyllene, caryophyllene oxide, total hop essential oils, hop oil fractions (PHA® Spicy, Citrusy, Floral, Herbal and Sylvan, Botanix, U.K.)). For this purpose, iso-α-acid bittered beers (see **section 2.2.7**) were aromatised with the SOP fraction (addition rate: 500 and 1000 µg/L). These beers were compared with reference beers (R) and beer with addition of the SHC fraction (addition rate: 500 µg/L). Additions of the SHC and SOP fractions to beer bottles were performed under nitrogen atmosphere (in absence of oxygen in an airlock closed workstation, Don Withney Scientific Limited). The beers were subsequently stored at 0°C for 24 h for equilibration prior to sensory evaluation. Two hours before sensory evaluation, beers were taken out of the refrigerator. Each panellist was served four beer samples: SOP (500 µg/L), SOP (1000 µg/L), SHC (500 µg/L), and the reference beer (R). Panellists were asked to score the intensity of pre-selected odour/aroma descriptors (malt/worty, fruity, floral, citrusy, hoppy, spicy, woody, hay/straw) on a scale ranging from 0 to 8 (0=not detectable, 8=very high intensity).

4.3 Results and discussion

4.3.1 Boiling of SHC fraction and detection of newly formed OSs

The sesquiterpene hydrocarbon (SHC) fraction was isolated from hop essential oil (cv. Saaz) with SPE. Next, the SHC fraction was analysed via HS-SPME-GC-MS for determination of its relative composition and (tentative) identification of volatiles (**Table 4-1**). The fraction comprised about 40 different compounds, of which 35 compounds were (tentatively) identified. SHCs represented 96% of the total peak area in the TIC chromatogram, which confirms our results obtained by GC-FID.

Table 4-1. Composition of the sesquiterpene hydrocarbon (SHC) fraction (90% EtOH fraction) obtained by SPE of hop essential oil cv. Saaz and subsequent HS-SPME-GC-MS. Relative amount based on relative peak areas (%); (tentative) identification based on reference compound (RC), mass spectrum (MS), and calculated retention index (RI).

compound	RI	relative amount (%)	identification
α-Cubebene / clovene	1337	0.07	MS. RI. RC /MS. RI. RC
Unknown (m/z 93,108)	1350	0.04	
α-Ylangene	1356	0.17	MS, RI
α-Copaene	1361	0.54	MS, RI, RC
Unknown (m/z 81,123)	1367	0.03	
7-cubebene	1373	0.06	MS, RI
10(9→8)Abeo-5,9-cyclo-(1R,5S,8S,9S)-caryophyll-3-ene	1377	0.05	MS, RI
7-*epi*-Sesquithujene	1380	0.04	MS, RI
Iso-caryophyllene	1389	0.09	MS, RI, RC
β-Caryophyllene	1405	13.88	MS, RI, RC
Caryophylla-4(12),8(13)-diene	1408	0.07	MS, RI
β-Copaene	1410	0.58	MS, RI
Aromadendrene	1419	0.05	MS, RI, RC
trans-α-Bergamotene	1430	1.11	MS, RI
α-Humulene	1448	47.80	MS, RI, RC
β-Farnesene	1460	25.15	MS, RI, RC
γ-Muurolene	1468	1.13	MS, RI
α-Amorphene	1469	0.26	MS, RI
β-Selinene	1472	0.40	MS, RI
Unknown sesquiterpene (m/z 69,93)	1473	0.16	
Unknown sesquiterpene	1475	0.04	
γ-Amorphene	1480	0.46	MS, RI
α-Selinene	1480	0.38	MS, RI
epi-Zonarene	1483	0.38	MS, RI
(Z,E)-α-Farnesene	1484	0.23	MS, RI
α-Muurolene	1485	0.39	MS, RI
δ-Amorphene	1489	0.09	MS, RI
γ-Cadinene	1496	1.78	MS, RI
(Z)-γ-Bisabolene	1497	0.33	MS, RI
trans-Calamenene	1499	0.43	MS, RI
Unknown sesquiterpene	1500	0.07	
δ-Cadinene	1507	2.61	MS, RI
trans-Cadina-1,4-diene	1513	0.20	MS, RI
Selina-4(15),7(11)-diene	1514	0.11	MS, RI
α-Calacorene isomer	1515	0.19	MS
α-Cadinene	1518	0.34	MS, RI
Selina-3(7),11-diene	1521	0.15	MS, RI
(E)-α-Bisabolene	1526	0.08	MS, RI
α-Calacorene	1531	0.04	MS, RI
Relative area of sequiterpene hydrocarbons		95.99	

Boiled dilutions of the SHC fraction (500 mg/L, boiling time 0-5 h) were analysed via HS-SPME-GC-MS, and the normalised areas of the four compound classes, as well as the total of detected volatiles, were calculated as a measure for the corresponding levels (see **Figure 4-3**).

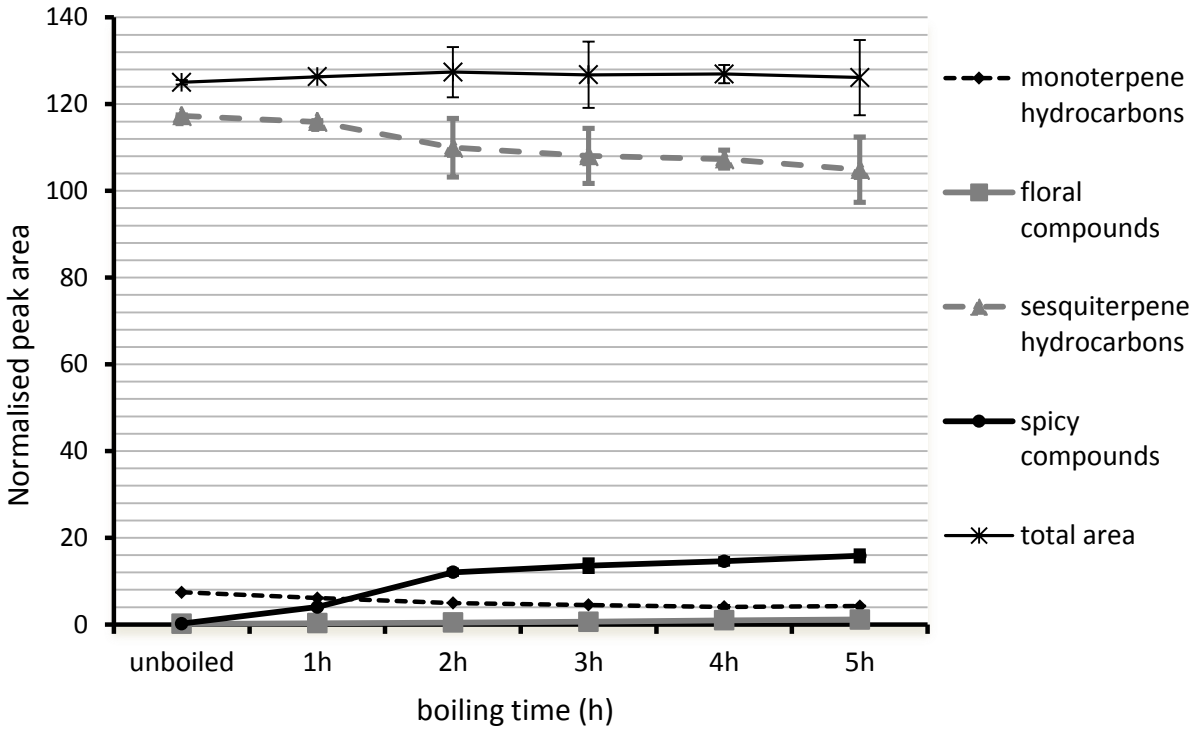


Figure 4-3. Normalised peak area (normalised for peak area of 2-heptanol as an internal standard) of monoterpene hydrocarbons, floral compounds, sesquiterpene hydrocarbons, spicy compounds and the total of hop volatiles in chromatograms of boiled SHC dilutions (500 mg/L) (n=2), obtained via HS-SPME-GC-MS analysis.

Clearly, the level of SHCs decreased with increasing boiling times, whereas a constant level was observed for the total peak area of the hop-derived volatiles. The level of the monoterpene compound class (present as 'impurities' of the total SHC fraction; < 1%), which includes α-pinene, β-pinene, β-myrcene and limonene, showed a decrease with increasing boiling times (recovery of 57% after 5 h of boiling, compared to the unboiled SHC fraction). No floral compounds were detected in the unboiled samples. However, two floral compounds were formed upon boiling of the SHC fraction. One of them is unknown (RI:1250), whereas the other compound was tentatively identified as perillene (RI:1088). When calculating the normalised areas of perillene and plotting these as a function of boiling time, a linear increase (R=0.9985) was observed (data not shown). Perillene, which is a cyclic oxygenated monoterpenoid (see **Figure 4-4**, structure (**a**)), may be an oxidation product formed when boiling β-myrcene in water under an oxygen-containing atmosphere, and its levels were previously found to increase upon boiling of total hop essential oil cv. Saaz (**Chapter 2**).

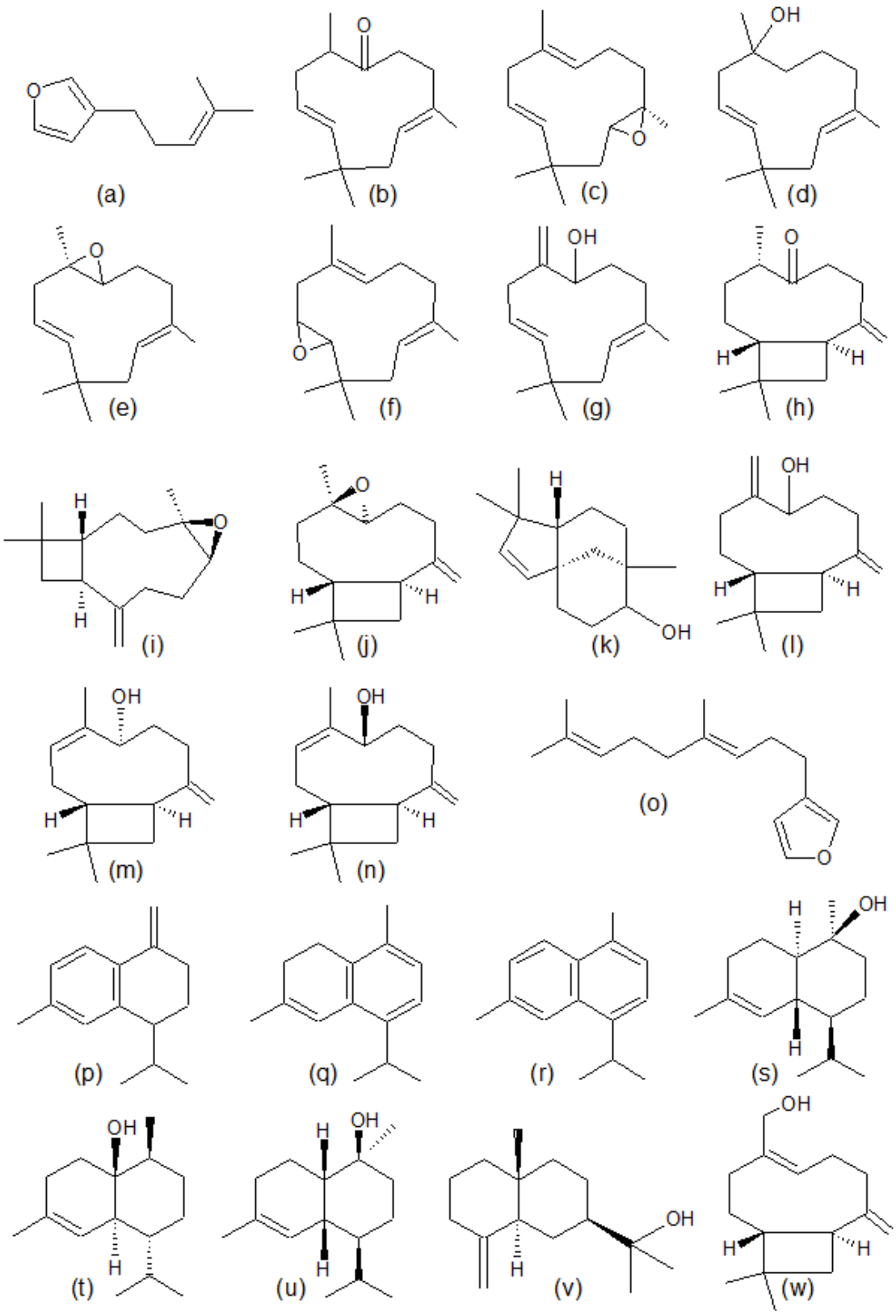


Figure 4-4. Structures of hop oil-derived compounds. (a) perillene, (b) humuladienone, (c) humulene epoxide I, (d) humulol, (e) humulene epoxide II, (f) humulene epoxide III, (g) humulenol II, (h) 4S-dihydrocaryophyllene-5-one, (i) isocaryophyllene epoxide A, (j) caryophyllene oxide, (k) covenol, (l) caryophylla-4(12),8(13)-diene-5-ol, (m) (3Z)-caryophylla-3,8(13)-diene-5α-ol, (n) (3Z)-caryophylla-3,8(13)-diene-5β-ol, (o) E-dendrolasin, (p) β-calacorene, (q) α-corocalene, (r) cadalene, (s) τ-cadinol, (t) cubenol, (u) τ-muurolol, (v) β-eudesmol, (w) 14-hydroxy-β-caryophyllene.

In **Figure 4-3**, increasing levels of spicy compounds (including OSs) coincide with a decrease in levels of SHCs, thus suggesting oxidation of SHCs into oxygenated derivatives. However, despite the apparently strong increase in the level of the spicy fraction, the relative area of this particular compound class remained limited to 13% after 5 h of boiling, implying that SHCs are not so easily oxidised and thus remain largely untransformed under the applied conditions. Among the compounds detected in the spicy fraction after 5 h of boiling, various α-humulene derivatives (*i.e.* humuladienone (**Figure 4-4**, structure (**b**)), RI 1553; humulene epoxide I (**c**), RI 1578; humulol (**d**), RI 1582; humulene epoxide II (**e**), RI 1589; humulene epoxide III (**f**), RI 1611; humulenol II (**g**), RI 1613) and β-caryophyllene derivatives (*i.e.* 4S-dihydrocaryophyllene-5-one (**h**), RI 1532; isocaryophyllene epoxide A (**i**), RI 1535; caryophyllene oxide (**j**), RI 1563; clovenol (**k**), RI 1564; caryophylla-4(12),8(13)-diene-5-ol (**l**), RI 1617; (3Z)-caryophylla-3,8(13)-diene-5α/β-ol (**m**)/(**n**), RI 1641/1657) were tentatively identified, clearly pointing to transformation of SHCs, in particular of α-humulene and β-caryophyllene, into OSs. In addition, we also detected E-dendrolasin (RI 1558, **Figure 4-4** (**o**)), a furan derivative in the C15 series whose structure and chemical reactivity are highly similar to perillene in the C10 series (41). Furthermore, three sesquiterpene hydrocarbons (*i.e.* β-calacorene (**Figure 4-4**, structure (**p**)), RI 1543; α-corocalene (**q**), RI 1599; cadalene (**r**), RI 1568) were detected. These SHCs were not present in the original SHC fraction and, therefore, our results confirm that SHCs are also converted into other sesquiterpenes. In agreement with results from the previous chapter (**Chapter 2**), we did not detect any cadinols (*e.g.* τ-cadinol (**Figure 4-4**, structure (**s**)), cubenol (**t**), τ-muurolol (**u**), β-eudesmol (**v**)) upon boiling, despite the presence of several cadinenes and selinenes in the unboiled SHC fraction.

4.3.2 Sensory evaluation of the SHC and SOP fraction in non-aromatised iso-α-acid-bittered beer

For sensory evaluation of the sesquiterpene oxidation products (SOPs) formed upon boiling, SPE fractionation was performed on the boiled SHC fraction to obtain an enriched oxygenated sesquiterpenoid fraction (SOP fraction). Prior to aromatising reference beers, four commercial lager beers were analysed in duplicate via HS-SPME-GC-MS to estimate the concentration of OSs in conventionally hopped beers. To this end, a caryophyllene oxide calibration curve (nine points, 0-1000 µg/L, R^2=0.9932, a= 27.037, b=-0.0205, where a is the slope and b is the intercept) was used to determine the level of OSs in beer in terms of caryophyllene oxide equivalents. The OS level in the different beers amounted to 87 ± 5 µg/L on average. Aiming at clearly pronounced aroma impressions of the SOP fraction in beer, a 5- and 10-fold increased addition rate (500 µg/L and 1000 µg/L, respectively) of the SOP

fraction was selected to aromatise pilot-scale lager beer, exclusively bittered with iso-α-acids extract.

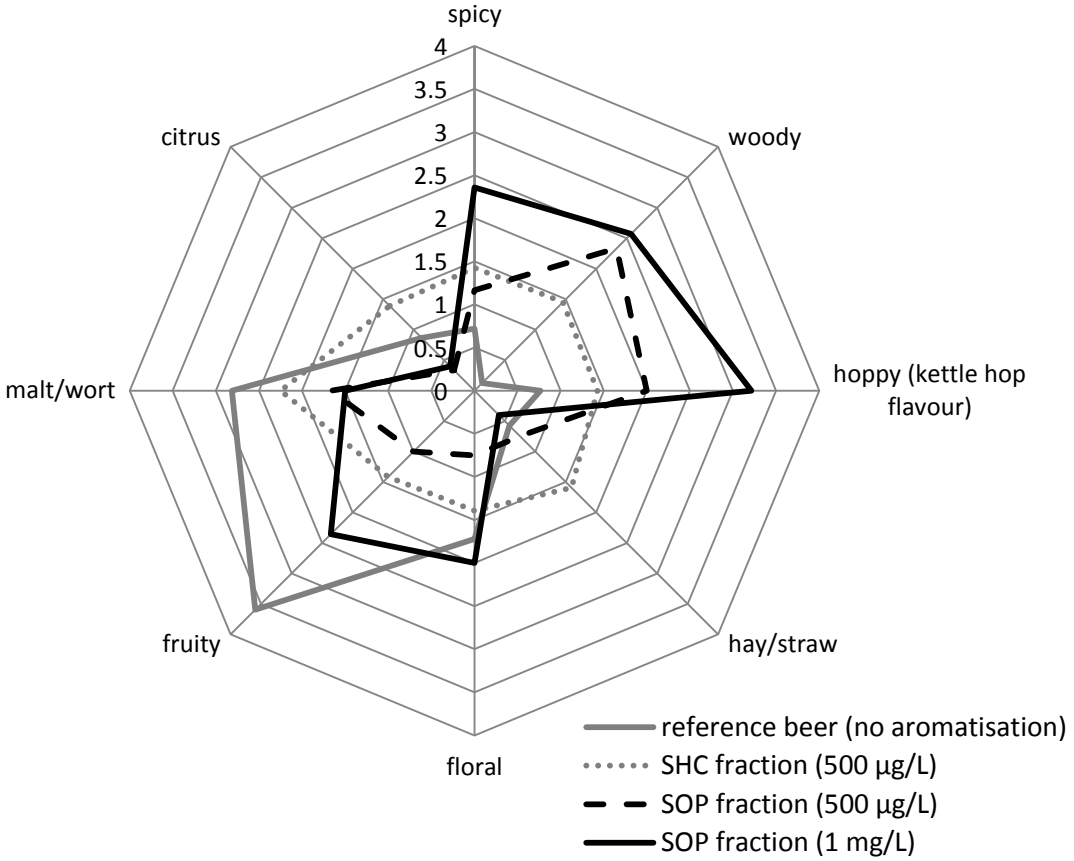


Figure 4-5. Spider plot displaying the average of individual scores (12 assessors) obtained via sensory evaluation of the sesquiterpene oxidation product (SOP) fraction and sesquiterpene hydrocarbon (SHC) fraction in non-aromatised iso-α-acid bittered beer.

The average scores for preselected descriptors used during sensory evaluation of the SHC and SOP fractions in beer by our taste panel are presented in a spider plot (**Figure 4-5**). The non-aromatised reference beer was scored relatively high for 'floral', 'fruity' and 'malt/wort' flavours. These descriptors were scored lower for the beer with addition of the unboiled SHC fraction (addition rate: 500 µg/L), whereas other aroma characteristics such as 'citrus', 'spicy', 'woody', 'hoppy' and 'hay/straw' were marked higher. Upon addition of the SOP fraction to the beer (addition rates: 500 µg/L and 1 mg/L, respectively), 'woody', 'hoppy' and 'spicy' scents were predominant in the flavour profile of the aromatised beers, in particular in the beer with the highest addition rate.

4.3.3 GC-O analysis for determination of odour impact compounds in the SOP fraction

Because addition of the SOP fraction to non-aromatised iso-α-acid bittered lager beer reduced 'malty/worty' flavours and increased 'woody/spicy/hoppy' notes, the SOP fraction was analysed via GC-O to determine the flavour-active compounds responsible. As displayed in **Table 4-2**, the enriched SOP fraction was mainly composed of α-humulene and β-caryophyllene oxidation products and derivatives thereof. **Table 4-2** shows 11 odour-active regions, and most of the compounds present in these intervals were (tentatively) identified. Two zones were clearly odour-active, as reflected by the detection frequencies of 8 and 10 out of the 10 analyses performed.

The first interval with obvious odour impact comprised humulene epoxide III, humulenol II and caryophylla-4(12),8(13)-diene-5α/β-ol, the olfactory sensation of the total interval being described as 'woody/green/hoppy'. Humulene epoxide III has already been associated with 'hoppy' aroma for several decades[17,28,84], and humulenol II has also been correlated with the herbal/spicy flavour of beer[10,113]. The third compound in this region was identified as caryophylla-4(12),8(13)-diene-5α/β-ol (identical RI and mass spectrum for α and β isomer). Deinzer and Yang[28] reported the presence of the β isomer in unprocessed hop oil cv. Hallertauer. In their study, caryophylla-4(12),8(13)-diene-5-β-ol was detected together with humulol and humulenol II in a hop oil fraction that was described as 'floral perfume', 'herbal', 'spicy' but also as 'noble'; in addition, this fraction was scored relatively high for European hoppy aroma. The α-isomer was found in hop oil cv. Saaz and Hallertauer by Yang *et al.*[85] and also Nance and Setzer[210] detected this compound in six different hop varieties. More specifically, Nance and Setzer[210] found the highest level of the α-isomer in two noble hop varieties (Saaz and Hallertauer); therefore, a correlation between the presence of this compound and 'noble' hoppy aroma might exist. Interestingly, the abovementioned compounds, in particular humulenol II and caryophylla-4(12),8(13)-diene-5α/β-ol, were also found to be odour-active in **Chapter 3** in which a commercial lager beer, exclusively kettle hopped with the German noble aroma hop varieties Hallertau Mittelfrüh and Tettnang Tettnanger, was fractionated and subsequently evaluated via GC-O.

Table 4-2. Composition and characterisation (including GC-O data) of the sesquiterpene oxidation product (SOP) fraction obtained by SPE of boiled hop oil sesquiterpene hydrocarbons (SHCs). a) Compounds with clear mass spectra, detected in SOP fraction. b) RI= Calculated retention index (RTX-1 capillary column) of components in SOP fraction. c) Relative area percentage (± stdev, n=3). d) Identification on basis of comparison of retention index (RI), of mass spectra (MS) and of authentic reference compounds (RC). e) Detection frequency in GC-O analysis (out of 10 analyses). f) Odour descriptors of flavour-active compounds, extracted from literature data. Compounds in grey box elute in flavour-active interval. Frame with dotted line indicates one flavour-active zone and comprises compounds identified within this interval. *= compound found upon acid catalysed rearrangement of humulene epoxide mixture. **= compound found upon epoxidation of α-humulene/β-caryophyllene/isocaryophyllene. ***= compound found upon photosensitised oxidation of α-humulene/β-caryophyllene.

Compound[a]	n°	RI[b]	Area %[c]			Identification[d]	DF[e]	Odour descriptor[f]
β-Caryophyllene	1	1415	0.06	±	0.01	RI, MS, RC		woody, spice[9]
Unknown (m/z 81, 95, 109, 123, 138, 149, 177, 191, 205, 220)	2	1439	4.85	±	0.42		4	
α-Humulene	3	1443	0.81	±	0.08	RI, MS, RC	6	woody[9]
β-Farnesene	4	1447	0.32	±	0.01	RI, MS, RC		woody[9]
Unknown (m/z 91, 117, 131, 145, 159, 187, 202, 220)	5	1456	0.44	±	0.05		4	
1,5,8,8-Tetramethyl-12-oxa-5-tricyclo[7.2.1.$0^{6,9}$]dodecene	6	1468	0.23	±	0.02	MS, *	4	lime, cedar[3]
Unknown (m/z 81, 95, 109, 123, 138, 149, 177, 191, 205, 220)	7	1476	2.90	±	0.25		3	
Unknown (m/z 95, 106, 119, 147, 162, 187, 202, 218)	8	1501	0.52	±	0.04			
Unknown (m/z 69, 163)	9	1510	8.58	±	0.29			
α-Calacorene	10	1525	0.37	±	0.02	RI, MS	4	woody[9]
4S-Dihydrocaryophyllene-5-one	11	1532	1.22	±	0.07	RI, MS		cedar, floral[4]
Isocaryophyllene epoxide A	12	1535	0.22	±	0.01	RI, MS, **	6	
4R-Dihydrocaryophyllene-5-one	13	1535	0.25	±	0.01	RI, MS		
6(5→4)-abeo-caryophyll-7-en-5-al	14	1539	0.19	±	0.01	RI, MS		
unknown (m/z 93, 107, 121, 149, 177, 205, 220)	15	1546	0.53	±	0.05			
Humuladienone / caryolan-1-ol	16 / 17	1553	3.86	±	0.38	RI, MS	4	flowery, fresh[8], hop-like[2], green[7] / green[7]
E-Dendrolasin / 6(5→4)-abeo-caryophyll-8(13)-en-5-al	18 / 19	1558	6.32	±	0.27	RI, MS		- / cedar, lemon[4]
Caryophyllene oxide	20	1563	0.61	±	0.01	RI, MS, RC, **		cedar, lime, floral[4]
Clovenol	21	1564	0.60	±	0.02	RI, MS		
Humulene epoxide I	22	1578	4.47	±	0.51	RI, MS, **		hay-like[1]
Humulol	23	1582	1.24	±	0.05	MS / RI, MS	4	hay-like[1]
Humulene epoxide II	24	1589	3.32	±	0.25	RI, MS, **		lime, cedar, rubber[3], moldy[1]
Humulene allylic alcohol	25	1598	1.09	±	0.06	MS, ***		
Humulene epoxide III	26	1611	12.67	±	0.35	RI, MS, **	8	lime, cedar, spicy, rubber[3]
Humulenol II	27	1613	8.95	±	0.62	RI, MS, ***		sagebrush[1], lime, cedar, pineapple[3]
Caryophylla-4(12),8(13)-diene-5α/β-ol	28	1617	2.47	±	0.19	RI, MS, ***		lime, herbal, rubber[4]
3Z-Caryophylla-3,8(13)-diene-5α-ol	29	1641	4.60	±	0.44	RI, MS, ***	10	cedar, lime, herbal, pine[4]
Unknown (m/z 79, 80, 81) / 14-hydroxy-β-caryophyllene	30 / 31	1646	6.13	±	0.10	/ RI, MS		- / cedar[5]
Unknown (m/z 69, 81, 93, 109, 159, 177)	32	1652	0.72	±	0.01			
Unknown (m/z 80, 93, 121, 137)	33	1653	0.56	±	0.05			
(3Z)-Caryophylla-3,8(13)-diene-5β-ol / cadalene	34 / 35	1657	0.74	±	0.04	RI, MS, *** / RI, MS		cedarwood[6] / -
(6Z)-Pentadecen-2-one	36	1660	1.15	±	0.05	RI, MS		
Humulene allylic alcohol Y	37	1666	3.27	±	0.11	MS, ***		

1=Fukuoka and Kowaka, 1983[139], 2= Shimazu *et al.*, 1975[16], 3= Yang *et al.*, 1993[84], 4= Yang *et al.*, 1993[85], 5= Eyres *et al.*, 2007[114], 6= Nielsen, 2009[146], 7= Van Opstaele *et al.*, 2013[124], 8= Lermusieau and Collin, 2001[7], 9= http://www.thegoodscentscompany.com/ (accessed January 29, 2015)

The second region with clear odour-activity was described as 'green' and 'woody' by the assessors and was composed of at least eight different compounds (see also **Table 4-2**), among which caryophylla-3,8-(13)-diene-5β-ol and 14-hydroxy-β-caryophyllene (**Figure 4-4**, structure (**w**)) were (tentatively) identified via mass spectral comparison and comparison of RIs. These results confirm literature data reported by Eyres *et al.*[114] and Nielsen[146], who respectively identified 14-hydroxy-β-caryophyllene and caryophylla-3,8-(13)-diene-5β-ol as potent 'woody/cedarwood' odour impact compounds in, respectively, hop essential oil and hop teas/ale beers. Thus, the strong cedarwood aroma was detected by both authors, although they assigned the odour to a different compound. In our study, we found evidence for the presence of both 14-hydroxy-β-caryophyllene and caryophylla-3,8(13)-diene-5β-ol, eluting close to each other in this particular odour-active region of the SOP fraction. Moreover, because the assessors recorded the start of the perceived odour at caryophylla-3,8(13)-diene-5α-ol, this compound is also considered to be flavour-active. Other compounds eluting in this region were, besides some unidentified compounds, cadalene and (6Z)-pentadecen-2-one. These two compounds and also 14-hydroxy-β-caryophyllene, were detected in hops by Nance and Setzer[210]. However, they found 14-hydroxy-β-caryophyllene and cadalene in only one of the three Saaz hop samples investigated in their study, whereas increased levels of (6Z)-pentadecen-2-one were observed in the same sample. This suggests oxidation in that specific Saaz hop sample, which is supported by our findings, demonstrating the presence of 14-hydroxy-β-caryophyllene, cadalene, and (6Z)-pentadecen-2-one after boiling (forced oxidation) of the Saaz-derived SHC fraction, and also by literature data on storage of hops as reported by Tressl *et al.*[78,82]. (6Z)-pentadecen-2-one was also previously detected by our research group in single-variety spicy hop essences[124], whereas cadalene and caryophylla-3,8(13)-diene-5β-ol were assigned to a flavour-active region of the spicy fraction of a commercial kettle hopped lager (**Chapter 3**).

Next to the two olfactometric regions discussed earlier, a series of zones showing lower detection frequencies were observed (see **Table 4-2**) in which α-humulene, 1,5,8,8-tetramethyl-12-oxa-5-tricyclo[7.2.1.$0^{6,9}$]dodecene (*i.e.* a humulene epoxide II/III hydrolysate[138]), α-calacorene, isocaryophyllene epoxide A, caryolan-1-ol, humuladienone and humulol were identified. Caryolan-1-ol, humuladienone and α-calacorene were also previously indicated as odour-active compounds in a varietal spicy hop essence[124] and were detected in an odour-active region of a commercial kettle hopped lager (see **Chapter 3**). Interestingly, α-calacorene was also proposed as a key compound for hop aroma characteristics in beer on the basis of principal component analysis (PCA) of GCxGC-TOF-MS (comprehensive two-dimensional gas chromatography coupled to a time-of-flight mass spectrometer) data for hop-derived compounds and organoleptic scores of hopped beers[183].

4.3.4 Caryophyllene derived alcohols: identification and flavour-activity

Because (tentative) identification of the caryophyllene derived allylic alcohols (compounds **28, 29, 34**, **Table 4-2**) in the SOP fraction is not straightforward (*e.g.* owing to co-elution and lack of authentic reference compounds), caryophyllene allylic alcohols were prepared via photosensitised oxidation of β-caryophyllene to obtain a mixture of reference compounds, providing high quality mass spectral information and RIs for our compounds of interest. The prepared mixture contained two caryophyllene-derived allylic alcohols, identified on the basis of RI, mass spectrum, and reaction product ratios found in the literature[211,212]. The first (eluting) is caryophylla-4(12),8(13)-diene-5α/β-ol, for which the RI and mass spectrum corresponds to compound **28** (**Table 4-2)**. The other allylic alcohol is (3Z)-caryophylla-3,8(13)-diene-5-ol, covering two isomers (α and β-isomer) with highly similar mass spectra (compounds A and D, see **Figure 4-6**) but different RIs. The RIs and mass spectra of the caryophyllene derived allylic alcohols (peak **29** and peak **34**, **Table 4-2**) in the SOP fraction coincided with those found for compound A and compound D, respectively, in the prepared allylic alcohol mixture and with those found in the literature[189].

For additional confirmation of the tentative identification of compound **31** (**Table 4-2**), we looked for plant material containing high levels of 14-hydroxy-β-caryophyllene. According to Demirci and co-workers[213,214], the essential oil from birch buds (*Betula* species) contains a relatively high level of 14-hydroxy-β-caryophyllene. Hence, *Betula* buds (250 mg) were ruptured by use of a mortar and pestle and analysed via HS-SPME-GC-MS. In the chromatographic area of interest, four caryophyllene derivatives with a highly similar mass spectrum were found. The first and last eluting compounds were easily identified as (3Z)-caryophylla-3,8(13)-diene-5α and β-ol, respectively, based on comparison of RIs and mass spectra with those obtained from analysis of the caryophyllene allylic alcohol mixture (**A** and **D**, see **Figure 4-6**). The second compound (compound **B**, see **Figure 4-6**), comprising the largest peak area, could be tentatively identified as 14-hydroxy-β-caryophyllene based on the mass spectrum and RI. The structure of 14-hydroxy-β-caryophyllene, reported by Barrero *et al.*[215] and Baser and Demirci[216], can also be found under the name 12-hydroxy-caryophyllene[189] (depending on at which carbon atom one starts to count). The latter nomenclature is consistent with the names we used for the allylic alcohols (caryophylla-4(12),8(13)-diene-5-ol and (3Z)-caryophylla-3,8(13)-diene-5-ol). However, because the structure of compound B is widely known as 14-hydroxy-β-caryophyllene in the literature, we preferred to use the latter nomenclature. In addition, this compound was also proposed to be identical to α-betulenol, whereas 14-hydroxy-isocaryophyllene would be equal to β-betulenol[216,217].

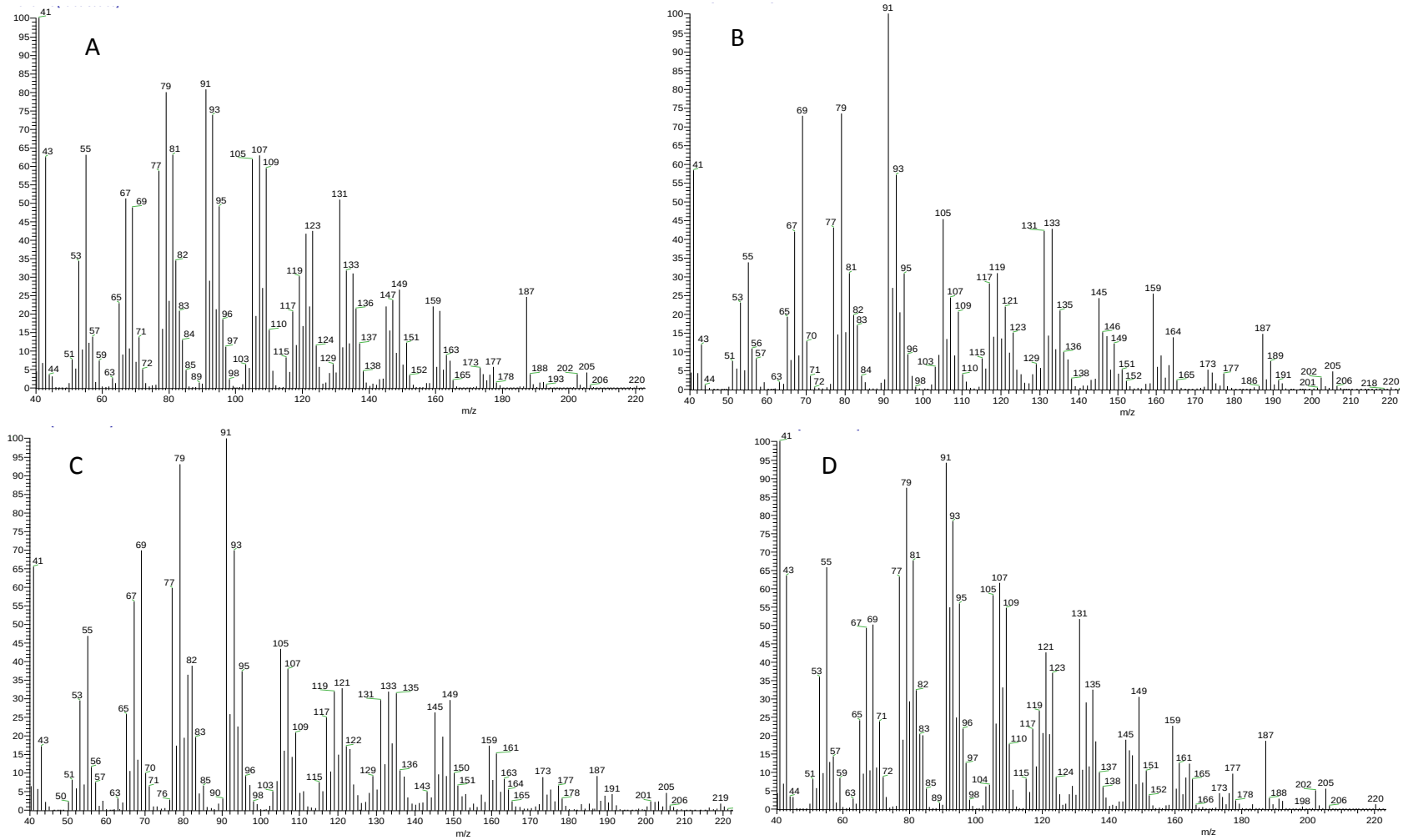


Figure 4-6. Experimental mass spectra for compounds A, B, C and D. The mass spectra of compounds A and D were derived from the caryophyllene allylic alcohol reference mixture and the mass spectra of compounds B and C from analysis of birch bud oil.

A= (3Z)-caryophylla-3,8(13)-diene-5α-ol, mass spectrum found (70 eV, two most intense ions each 14 mass units above m/z 40): 41 (100), 43 (61), 55 (63), 67 (52), 79 (81), 81 (63), 91 (83), 93 (72), 105 (64), 107 (64), 121 (43), 123 (44), 131 (52), 133 (33), 147 (25), 149 (28), 159 (23), 161 (22), 173 (6), 177 (6), 187 (26), 188 (4), 202 (4), 205 (5), 220 (1).

B= 14-hydroxy-β-caryophyllene, mass spectrum found (70 eV, two most intense ions each 14 mass units above m/z 40): 41 (58), 53 (23), 55 (34), 67 (42), 69 (73), 79 (73), 91 (100), 93 (57), 105 (45), 107 (24), 117 (28), 119 (31), 131 (42), 133 (43), 145 (24), 146 (15), 159 (25), 164 (14), 173 (5), 174 (4), 187 (15), 189 (7), 202 (3), 205 (5), 220 (1).

C= 14-hydroxy-caryophyllene isomer, mass spectrum found (70 eV, two most intense ions each 14 mass units above m/z 40): 41 (66), 53 (29), 55 (47), 67 (56), 69 (70), 79 (93), 91 (100), 93 (70), 105 (43), 107 (38), 119 (32), 121 (33), 133 (32), 135 (31), 145 (26), 149 (30), 159 (17), 161 (15), 173 (9), 177 (7), 187 (10), 191 (4), 201 (2), 205 (5), 219 (1), 220 (1).

D= (3Z)-caryophylla-3,8(13)-diene-5β-ol, mass spectrum found (70 eV, two most intense ions each 14 mass units above m/z 40): 41 (100), 43 (64), 55 (66), 67 (49), 79 (88), 81 (68), 91 (95), 93 (79), 105 (58), 107 (62), 121 (43), 123 (38), 131 (52), 135 (33), 145 (19), 149 (31), 159 (23), 161 (13), 173 (4), 177 (10), 187 (19), 188 (3), 202 (5), 205 (6), 220 (1).

Interestingly, we detected a minor compound in birch bud oil, eluting closely after 14-hydroxy-β-caryophyllene and showing a highly similar mass spectrum (compound **C**, see **Figure 4-6**). This compound is probably a 14-hydroxy-β-caryophyllene isomer, and although there is some confusion regarding the stereochemistry and nomenclature of 14-hydroxy-β-caryophyllene isomers[114,188,189], there are clearly four isomers because each caryophyllene isomer (*i.e.* Z-caryophyllene = iso-caryophyllene = *cis*-caryophyllene; β-caryophyllene = *trans*-caryophyllene = E-caryophyllene; 9-epi-iso-caryophyllene; 9-epi-caryophyllene) may give rise to a corresponding 14-hydroxy-isomer (*i.e.* 14-hydroxy-iso-caryophyllene,14-hydroxy-β-caryophyllene, 14-hydroxy-9-epi-iso-caryophyllene, and 14-hydroxy-9-epi-(E)-caryophyllene, respectively).

To verify flavour-activity of the caryophyllene alcohols detected in the SOP fraction, GC-O was performed on both the prepared caryophyllene allylic alcohol mixture and the extract from birch buds. The detection frequencies of each compound are displayed in **Table 4-3**. During the birch bud analyses, two assessors could determine individual flavour-active peaks, whereas the third assessor was oversensitive and systematically detected one broad flavour-active interval. This same assessor also detected both allylic alcohols in each analysis of the allylic alcohol reference mixture. Clearly, as was also reported by others[146], a significant difference exists toward sensitivity of assessors for caryophyllene derived alcohols. In conclusion, flavour-activity of both 14-hydroxy-β-caryophyllene and (3Z)-caryophylla-3,8(13)-diene-5β-ol was confirmed, and even the isomer of the caryophyllene-derived allylic alcohol ((3Z)-caryophylla-3,8(13)-diene-5α-ol) and caryophylla-4(12),8(13)-diene-5α/β-ol proved to express odour upon GC-O analysis.

Table 4-3. Detection frequencies of compounds, determined by GC-O analysis (3 assessors, analysis in triplicate) on the caryophyllene allylic alcohol reference mixture (DF^a) and the birch buds extract (DF^b), respectively. Identification on the basis of preparation of the particular compound via photosensitised oxidation of β-caryophyllene (*), comparison of retention indices (RIs) and comparison of mass spectra (MS).

Caryophyllene derived alcohol	DF^a	DF^b	identification
Caryophylla-4(12),8(13)-diene-5α/β-ol	7/9		RI, MS, *
(3Z)-Caryophylla-3,8(13)-diene-5α-ol	6/9	5/9	RI, MS, *
14-Hydroxy-β-caryophyllene		7/9	RI, MS
14-Hydroxy-caryophyllene isomer		3/9	RI, MS
(3Z)-Caryophylla-3,8(13)-diene-5β-ol	6/9	5/9	RI, MS, *

To summarise, the most obvious flavour-active zones in the SOP fraction, as detected via GC-O, were described as 'woody/hoppy' (first zone) and 'woody' (second zone), and clearly the same odour characteristics came to expression when adding the SOP fraction to beer. A spicy hop note in beer is associated with noble or kettle hop aroma[27], and it was found previously that the spicy hop flavour impression was related to the hop oil-derived OS fraction[19], which was confirmed by our current investigation. However, the precise identity of OSs responsible for this unique spicy hop-derived impression is still unclear. Humulene oxidation products and, in particular, humulene epoxides have been proposed to be important contributors to the 'herbal/spicy' and 'kettle-hop' flavour[10,17]. However, flavour-activity of these compounds has been questioned by several researchers, since the epoxides could not be detected via GC-O or did not exhibit spicy or hoppy flavour[26,28,83,139]. Next, the focus shifted to the humulene epoxide and caryophyllene oxide hydrolysis products[84,85,138,174], which were proposed to be at least partly responsible for a herbal/spicy aroma. In the SOP fraction studied here, as well as in our previous chapter (**Chapter 3**), humulenol II appeared to be potentially relevant with respect to flavour-activity. Furthermore, caryophyllene hydrolysis and rearrangement products, in particular the allylic alcohols, have been associated by others[114,146] with the 'woody' note and this was clearly confirmed in the present study. It was not possible to specifically determine one or more hop oil-derived volatiles as being directly responsible for hoppy aroma of beer. Nevertheless, addition of the SOP fraction to non-aromatised beer clearly imparted 'hoppy' aroma and, therefore, it should be taken into consideration that 'hoppy' aroma could be the result of additive and/or synergetic effects among several flavour-active OSs.

4.4 Conclusions

In this chapter, the applied innovative approach (*i.e*. isolation of SHCs from total hop oil, boiling and isolation of the formed oxidation products) allowed us to unambiguously prove that several sesquiterpene oxidation products (SOPs) are formed *de novo* upon boiling by oxidation of SHCs, and, for the first time, to demonstrate a cause-and-effect relationship between the presence of these compounds in lager beer and 'hoppy/spicy' aroma. These findings clarify the increase in 'spicy/hoppy' notes upon addition of boiled hop essential oil (cv. Saaz) to iso-α-acid-bittered lager beer, as observed in **Chapter 2**.

We were also able to propose key candidate impact compounds for the 'spicy', 'woody' and 'kettle hop' flavour of the SOP fraction (*i.e.* humulene epoxide III, humulenol II, caryophylla-4(12),8(13)-diene-5α/β-ol, (3Z)-caryophylla-3,8(13)-diene-5α/β-ol, 14-hydroxy-β-caryophyllene) and, except for humulene epoxide III, these compounds were previously found by us in flavour-active zones of the spicy fraction derived from a commercial beer that was kettle hopped with noble hop varieties (**Chapter 3**). Moreover, we have extended our approach beyond hops and brewing and confirmed the flavour-activity of caryophyllene-derived alcohols by performing GC-O on caryophyllene allylic alcohols, present in a chemically synthesized mixture and in *Betula* buds, respectively.

Basically, both the results of our previous chapters and results from others[114,146], as well as findings obtained in this study, clearly suggest particular OSs as lead components involved in spicy and hoppy aroma of beer. Together these findings may represent a clue to the identification of the true nature of hoppy aroma of beer and further support the relevance of boiling of aroma hops for development of 'kettle hop/ hoppy' aroma in real brewing practice.

Chapter 5

HEAT-INDUCED CHANGES IN THE COMPOSITION OF VARIETAL HOP ESSENTIAL OILS VIA WORT BOILING ON LAB SCALE

Chapter 5 corresponds to:

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Heat-induced changes in the composition of varietal hop essential oils via wort boiling experiments on lab scale.

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Investigation of the impact of boiled hop oil (cv.Saaz) concentration on recovery of different compound classes. Chemical-analytical profiling of varietal hop essential oils, boiled in wort on a lab scale. GC-O for determination flavour-active volatiles in hop oil (cv. Saaz), boiled in wort.

Tatiana Praet performed the experiments. The final manuscript was written by Tatiana Praet an revised and adapted after critical input by Prof. Luc De Cooman and Dr. Filip Van Opstaele. The authors are also grateful to Dr. G. Organ (Lion Nathan, Silverwater, Australia) for his helpful suggestions regarding boiling of a sesquiterpene hydrocarbon fraction cv. Super Pride.

5 HEAT-INDUCED CHANGES IN THE COMPOSITION OF VARIETAL HOP ESSENTIAL OILS VIA WORT BOILING ON LAB SCALE

5.1 Introduction

The 'early' addition of 'noble type' European aroma hops to the boiling kettle is associated with 'kettle hop' flavour[27] and, in general, such aroma hops tend to contain relatively high levels of α-humulene[27,28,84,105,106]. Moreover, hop essential oils rich in sesquiterpene hydrocarbons (SHCs) tend to express earthy, herbal, woody or spicy aromas[210] and high humulene to caryophyllene ratios (>3) are consistent with a European hoppy aroma[28,106]. During ageing of hops, SHCs are oxidised into SOPs[10,17,82] and, consequently, the ratio of humulene to humulene and its epoxides is a good indicator for the freshness status of hop samples[79]. For example, α-humulene is converted into humulene epoxide I and II[17,82] during ageing of hops and, interestingly, spicy hop flavour has been linked to humulene epoxide levels in beer[18,160].

Oxygenated sesquiterpenoids (OSs) (in particular oxidation products and their hydrolysed derivatives (SOPs)) are found in hop essential oil as a result of hop oxidation during drying and subsequent storage. Extraction of the oxygenated sesquiterpenoid fraction from total hop oil (using Solid Phase Extraction) and addition to beer imparts a spicy/herbal note reminiscent of 'noble' hop aroma[19]. Also Deinzer and Yang[28] fractionated hop oil (cv. Hallertauer Mittelfrüh), leading to various fractions (comprising humulene and caryophyllene derived alcohols) which were scored relatively high for European hop aroma, although the oil had not undergone kettle boil and 'noble' aroma was not expected.

Besides the chemical transformations in hops as such, it is assumed that the brewing process generates new hop-derived volatiles[83] and that oxidation of SHCs also occurs when boiling hop products in the kettle[10,16,39]. Several researchers identified a large series of α-humulene and β-caryophyllene epoxides and their derivatives, *i.e.* rearrangement and hydrolysis products, upon lab scale boiling of reference compounds[17,27,85,138] and hop oil fractions[135,139] in model solutions. Many of these volatiles were also detected in lager beers and, if the concentration in beer exceeds its flavour threshold, were also proposed to contribute to 'kettle hop' aroma[84,85,113]. In **Chapter 2**, an increase in the level of OSs upon lab scale boiling of hop essential oil (cv. Saaz) in water was proven, which was mainly due to an increase in levels of particular α-humulene and β-caryophyllene derivatives (*i.e.* humuladienone, caryophyllene oxide, humulene epoxide I-III, caryophylla-4(12),8(13)-diene-5-ol, (3Z)-

caryophylla-3,8(13)-diene-5β-ol, humulol). We were also able to pinpoint compounds, newly formed upon boiling of total hop essential oil and, apparently, the number of compounds detected exclusively in boiled hop oil increased as the boiled hop oil concentration increased. Among these compounds, some were previously detected in commercial kettle hopped lagers (*i.e.* 4-S-dihydrocaryophyllene-5-one, 1,5,8,8-tetramethyl-12-oxa-5-tricyclo[7.2.1.$0^{6,9}$]dodecane, 6(5→4)-abeo-8,12-cyclo-caryophyllan-5-al, humulol, an humulene allylic alcohol and an unidentified oxygenated sesquiterpenoid (m/z 93, 205, 220)) (see **Chapter 3**), thus providing evidence for generation of new compounds during the brewing process. Interestingly, addition of boiled hop essential oil (cv. Saaz) to a non-aromatised iso-α-acid-bittered lager beer reduced 'malty/worty' flavours and increased 'spicy' and 'hoppy' notes (see **Chapter 2**).

Therefore, in **Chapter 4**, we focused on hop-derived SOPs and their potential relation to 'hoppy' aroma of beer. By boiling of a varietal SHC fraction (cv. Saaz) in pure water and subsequent SPE-isolation of the oxidation products formed, we obtained an oxygenated fraction mainly consisting of SOPs. This fraction was admittedly created offline (*i.e.* outside the brewery) but, nevertheless, proved to be promising towards brewery applications since it imparted 'hoppy', 'spicy' and 'woody' flavours to a non-aromatised iso-α-acid-bittered lager beer. Aiming at determination of the compounds responsible for these perceived flavours, the sensory relevant SOP fraction was further subjected to GC-O analyses, revealing two pronounced flavour-active zones, a first zone comprising humulene epoxide III, humulenol II and caryophylla-4(12),8(13)-diene-5-ol and a second zone comprising (3Z)-caryophylla-3,8(13)-diene-5-ol (α and β) and 14-hydroxy-β-caryophyllene. Moreover, these volatiles (*i.e.* humulenol II, caryophylla-4(12),8(13)-diene-5α/β-ol, (3Z)-caryophylla-3,8(13)-diene-5α/β-ol, 14-hydroxy-β-caryophyllene) also eluted in flavour-active zones of the spicy fraction derived from a commercial beer, kettle hopped with noble hop varieties (see **Chapter 3**), which supports the hypothesis that (some of) these compounds may be lead components for 'kettle hop' aroma of beer.

In this chapter, boiling experiments (lab scale) with hop essential oil in wort are performed to investigate as to what extent our previous findings obtained in water also apply when boiling essential oil in wort. Finally, the potential of aroma hops to impart 'kettle hop' aroma is researched by performing lab scale boiling experiments in wort with a selection of pure varietal hop essential oils (cv. Saaz, cv. Hallertau Tradition, cv. Hallertau Perle vs. the bitter hop cv. Hallertau Magnum).

5.2 Experimental

5.2.1 Reference compounds

The following reference compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA) or MERCK (Darmstadt, Germany) and were of analytical grade: (-)-caryophyllene oxide (≥98.5%); 2-heptanol (98%); 2-undecanone (99.0%); ocimene (≥90.0%, mixture of isomers); p-cymene (≥99.0%); α-humulene (≥98.0%); β-myrcene (≥95.0%); ethyl *trans*-4-decenoate (97%). Ethanol (EtOH) absolute (≥99.8%) was purchased from VWR International (Zaventem, Belgium)). Iso-caryophyllene and oxygenated sesquiterpenoid mixtures of reference compounds were prepared as described in **section 2.2.1.2**.

5.2.2 Plant material

Hop essential oil was extracted from hop pellets T90 cv. Saaz, Hallertau Tradition, Hallertau Perle and Hallertau Magnum (crop year 2013), kindly provided by the Barth-Haas Group (Joh. Barth & Sohn GmbH & Co. KG, Nürnberg, Germany), and from hop pellets T90 cv. Super Pride, kindly provided by Dr. G. Organ (Lion Nathan, Silverwater, Australia). Pellets (100 g) were vacuum packed in laminated foils with an aluminum layer as a barrier to prevent oxygen diffusion and stored in the freezer (-18°C) to avoid oxidative degradation of hop oil compounds. Prior to extraction, 50 g pellets were disrupted using an electric coffee grinder (Krups 75) to facilitate subsequent extraction. Hop essential oil was extracted as described in **section 2.2.3.1**.

5.2.3 Preparation of sweet wort

Sweet wort (unboiled and unhopped) was prepared in our pilot brewery (2 hL scale). Brewing was performed with 40 kg milled pilsner malt and 140 L reverse osmosis brewing water with $CaCl_2$ added (80 mg/L) to adjust the pH to 5.25. The mashing-in scheme was as follows: 30 minutes at 63°C, 10 minutes at 72°C, 1 minute at 78°C. Next, the wort was filtered in a lauter tun and collected in the kettle (pH: 5.25; gravity: 12°P). Wort samples were taken from the kettle and collected in plastic containers (0.5 L), which were stored in the freezer (-18°C) until further use.

5.2.4 Boiling process

Hop oil dilutions cv. Saaz with code B or U_b were boiled in the incubation oven of the CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland); oven temperature: 100°C; stirring of the samples: 500 rpm, 5 seconds on, 2 seconds off. After 1h of boiling, the vials were removed and cooled in the cooler (3°C) of the CombiPAL autosampler. The samples with code U_u remained unboiled.

5.2.5 Experimental design of boiling experiments

5.2.5.1 Preparation of samples for boiling of hop essential oil (cv. Saaz) in MQ-water and subsequent dilution for HS-SPME-GC-MS analysis

A series of hop essential oil (cv. Saaz, $\rho_{hop\ oil}$: 868 g/L) dilutions in EtOH were prepared using volumetric flasks, resulting in hop oil stock solutions with different concentrations (see **Table 5-1 A**). For each different stock solution, four dilutions in MQ-water were prepared in a HS-SPME vial (Chromacol, clear glass, 20 mL, Welwyn Garden City, UK) which was closed with a bimetal magnetic crimp cap containing a silicone/Teflon septum (Interscience, Louvain-la-Neuve, Belgium). Of the four dilutions, three were boiled (code B: B_1, B_2, B_3) as described above and one remained unboiled (code U_u).

For HS-SPME-GC-MS analysis (in duplicate: n=2), all boiled and unboiled hop oil dilutions were further diluted to an identical concentration (aiming at 0.01 g/L as optimum hop oil concentration for HS-SPME-GC-MS analysis) to enable comparison. However, hop oil compounds are poorly soluble in water and might therefore cluster, leading to unrepresentative samples. To facilitate and increase the reproducibility of sampling, the solubility of the hop oil compounds was increased by addition of large volumes of EtOH. Therefore, all samples (both B and U_u) were opened and 5 mL EtOH was added to the aqueous hop oil dilutions. These 50/50 EtOH/hop oil in MQ-water dilutions (v/v) were subsequently further diluted in water. EtOH and internal standard (2-heptanol, 20 µL of a 12.5 g/L stock solution) were added in order to obtain equal EtOH concentrations (9.40%), internal standard concentrations (0.049 g/L) and hop oil concentrations (0.009 g/L) in all vials. For the hop oil samples of 0.01 g/L, no EtOH was added post-boil. Addition of 22.5 µL internal standard stock solution, 499.5 µL EtOH and 30 µL MQ-water to these samples resulted in an identical EtOH, internal standard and hop oil concentration (resp. 9.40%, 0.049 g/L and 0.009 g/L) for analysis. All dilution factors and calculated concentrations (weights taken into account) are summarised in **Table 5-1 A**.

5.2.5.2 Preparation of samples for boiling of hop essential oil (cv. Saaz) in sweet wort and subsequent dilution for HS-SPME-GC-MS analysis

To prepare a series of unboiled and boiled hop essential oil dilutions (cv. Saaz) in sweet wort, the procedure described above was employed (see **Table 5-1 B** for dilution factors and hop oil concentrations). Next to the reference for unboiled hop oil diluted in unboiled wort (U_u), an additional reference for unboiled hop oil in boiled wort was prepared (code U_b) for each stock solution by boiling sweet wort and post-boil addition of hop oil stock solution via injection with a syringe (100 µL, Hamilton, Reno, USA) through the septum of the cap (resulting in hop oil concentrations of 10 g/L, 5 g/L, 1 g/L, 0.5 g/L, 0.1 g/L and 0.01 g/L, total

volume 5 mL). All samples were further diluted for HS-SPME-GC-MS analysis (n=2) as described above.

5.2.5.3 Preparation of samples for boiling of hop essential oil (cv. Saaz, Hallertau Tradition, Perle and Magnum) in sweet wort and subsequent dilution for HS-SPME-GC-MS analysis

Hop essential oil (cv. Saaz, Hallertau Tradition, Perle and Magnum) dilutions in wort were prepared by dilution of hop essential oil in sweet wort, resulting in a final hop oil concentration of 10 g/L (total volume 5 mL) (see **Table 5-1 C** for dilution factors and hop oil concentrations). For each variety, two dilutions were prepared. One sample remained unboiled (code U_u) whereas the other sample was boiled (code B). Also an additional reference (code U_b) was prepared as described above. Next, all vials were diluted for HS-SPME-GC-MS analysis (n=2) as described previously (resulting in EtOH, internal standard and hop oil concentrations of resp. 9.40%, 0.049 g/L and 0.009 g/L, see **Table 5-1 C**).

Table 5-1. Preparation of hop oil dilutions in water or wort for boiling experiments and subsequent HS-SPME-GC-MS analysis. C= concentration.

	A: Boiling hop essential oil in MQ-water B: Boiling hop essential oil in wort (concentration dependency)						**C: Boiling hop essential oil in wort (varietal dependency)**
Preparation of hop oil stock solutions:							
Hop variety	Saaz	Saaz	Saaz	Saaz	Saaz	Saaz	Tradition, Perle, Magnum, Saaz
Dilution factor	undiluted	2	20	100	200	1000	undiluted
C hop oil stock solution (g/L)	868	440	88.7	44.2	8.74	0.857	868
Preparation of samples to be boiled:							
Dilution factor	86.8	88.0	88.7	88.3	87.4	85.8	86.8
Final hop oil C (g/L)	10.0	5.00	1.00	0.50	0.10	0.01	10.0
Post-boiling dilution:							
Dilution factor	2	2	2	2	2	1	2
Final hop oil C (g/L)	5.0	2.50	0.50	0.25	0.05	0.01	5.0
Preparation of samples for HS-SPME-GC-MS analysis:							
Dilution factor	556	278	55.6	27.8	5.56	1.11	556
Final EtOH v/v%	9.40						9.40
Internal standard C (g/L)	0.049						0.049
Final hop oil C (g/L)	0.009						0.009

5.2.6 Calculation of recoveries of compound classes upon boiling

The volatile profile of unboiled and boiled hop essential oil (cv. Saaz) dilutions in water or wort was characterised via HS-SPME-GC-MS analysis. The detected volatiles were subdivided in monoterpene hydrocarbons, floral compounds (incl. oxygenated monoterpenoids, aliphatic and branched esters, alcohols, ketones, aldehydes), SHCs and spicy compounds (mainly OSs and aliphatic/branched esters, alcohols, ketones and aldehydes), according to their chemical structure and the odour that these compound groups impart when isolated from total hop essential oil via SPE[15,184]. Specific screening of chromatograms for OSs was achieved via selected ion monitoring (post-analysis, selection of m/z 202, 205, 218, 220, 222 and 236). Peak areas of compound classes were normalised by taking the internal standard peak area and calculated concentration into account to compensate for variation in the HS-SPME extraction. Recoveries of compound classes upon boiling were calculated on the basis of these normalised peak areas (average of 2 duplicates). The standard deviation of the recovery upon boiling is an indicator for the reproducibility of the boiling process (boiling process in triplicate: B_1, B_2 and B_3). Concentration effects are investigated by plotting average recoveries as a function of the boiled hop oil concentration. For the series of samples in wort, an additional reference (U_b; unboiled hop oil in boiled wort) was employed to examine the degree of adsorption of hop oil to trub, formed during wort boiling. Changes in the wort-derived volatile profile upon boiling do not interfere in the hop oil fingerprint, as the use of a PDMS fiber favors extraction of non-polar hop oil volatiles and wort was diluted with EtOH and water prior to HS-SPME-GC-MS analysis.

5.2.7 HS-SPME-GC-MS analysis of unboiled and boiled hop essential oil for comparison of the volatile profiles

Hop-derived volatiles were extracted via headspace solid-phase microextraction (HS-SPME) (fibre coating: polydimethylsiloxane (PDMS), extraction time: 45 min, extraction temperature: 60°C, splitless injection) as previously described in **section 2.2.6**. Gas chromatographic conditions for separation of the volatiles were described in **section 2.2.6**. In this study two different oven programs were used for separation of the volatiles via the RTX-1 capillary column (nonpolar fused silica column, dimensions: 40 m x 0.18 mm x 0.25 µm): (1) 3 min at 35°C, temperature increase of 6°C/min to 250°C and hold of 5 min, resulting in a total acquisition time of 44 min. This program is used when the aim is determining peak areas of compound classes rather than accurate determination of the level of individual compounds. (2) 40°C (hold 1 min), ramp of 10°C/min (up to 70°C, for 1 min), ramp of 2°C/min (up to 137°C, hold 1 minute), ramp of 1°C/min (up to 172°C, hold 1 min), final ramp of 10°C/min (up to 250°C, hold 3 minutes). This second oven program resulted in

a total acquisition time of 85 minutes. This program is used for optimal separation of volatiles eluting in the spicy region. Mass spectrometric detection of volatiles was performed as described in **section 2.2.6**.

5.2.8 GC-olfactometry

Boiled hop oil cv. Saaz (10 g/L) dilutions in wort were diluted for HS-SPME-GC-MS analysis and screened for flavour-active constituents by GC-olfactometry as described in **section 3.2.7**. Three trained assessors were asked to sniff the sample in triplicate and to indicate flavour-active zones as well as to record the duration of the odour perception, which was achieved by using a handheld control unit with cursor wheel for signal generation. Assessors were thoroughly trained for odour detection and description of OSs using total hop essential oils (both boiled and unboiled), spicy fractions (prepared as described by Van Opstaele and coworkers[15,124,184] and mixtures of OSs that were obtained via chemical treatment of α-humulene and β-caryophyllene (see **section 2.2.1.1**). Olfactory global analysis (OGA) was applied to determine the significance of the detected flavour (the detection frequency (DF) indicates how many times the odourant was detected out of the 9 analyses).

5.2.9 Multivariate data analysis

Principal component analysis (PCA) and unsupervised cluster analysis (CA) were performed on the HS-SPME-GC-MS-derived normalised peak areas of compound classes in unboiled and boiled hop essential oil solutions. A demo of Solo 7.5 (R7.5.2) (Eigenvector Research, Inc., Manson, WA, USA) was used for PCA. This software equips users to perform multivariate analyses and includes the PLS Toolbox (chemometric multivariate analysis tools for use within the MATLAB® computational environment) graphical user interfaces. Prior to PCA, the data was automatically scaled and 2 principal components were selected. Unsupervised cluster analysis (CA) was performed using Minitab via Ward's method algorithm.

ANOVA (analysis of variance) was used to analyse statistically significant differences between average recoveries (n= 3) of different compound classes upon boiling of total hop essential oil in water/wort at different concentrations. In a first test, the impact of the reference (U_u or U_b) (factor A) and hop oil concentration (factor B) on the recovery when boiling total hop oil in wort was investigated separately for the different compound classes. In a second test, the impact of the matrix (water or wort) (factor A) and hop oil concentration (factor B) on the recovery of compound classes upon boiling of total hop oil was investigated. Two-way ANOVA analyses were performed using Minitab 17 software. For all analyses, a confidence level of 0.95 was selected (α-level: 0.05). P-values lower than 0.05 point to a statistical significant difference, thus indicating that the investigated factor has an impact on the recovery of the compound class.

5.3 Results and discussion

5.3.1 Effect of hop oil concentration on the recoveries of compound classes upon boiling

5.3.1.1 Boiling of hop essential oil (cv. Saaz) in MQ-water

In order to investigate the potential effect of the hop oil concentration on quantitative changes of compound classes, the average recoveries (n=3) for the different compound classes upon boiling in MQ-water are plotted as a function of the boiled hop oil concentration in . For low boiled hop oil concentrations, levels of chemical compound classes in unboiled and boiled hop oil do not significantly differ. For example, at the lowest boiled hop oil concentration (0.01 g/L), recoveries of all compound classes are close to 100%. However, as the boiled hop essential oil concentration is increased (up to 10 g/L), recoveries change significantly. Recoveries of total hop essential oil and sesquiterpene- and monoterpene hydrocarbons show a fast decrease as a function of increasing hop essential oil concentrations. The behaviour of terpene hydrocarbons can be rationalised by polymerisation[39,82,90,136,218] and chemical conversions (see **Chapter 2** and **4**) during the boiling process, which may become more significant at elevated hop oil concentrations. For the recoveries of the floral compounds, no relation with the initial hop oil concentration is found. Their higher solubility in polar matrices, due to the presence of at least one oxygen atom in their structural formula, may explain their limited losses during boiling in comparison to terpene hydrocarbons. A remarkable increase of the recovery upon boiling with increasing hop oil concentrations is observed for the spicy compounds. OSs in particular are characterised by an even more pronounced increase. A linear relationship between their recovery and the boiled hop oil concentration (a= 0.0135, b= 106.52, R^2= 0.9924) suggests that even higher yields of oxygenated sesquiterpenoids may be reached at elevated hop oil concentrations. The increase in the level of oxygenated sesquiterpenoids can be explained by *de novo* formation, i.e. by chemical oxidation of sesquiterpene hydrocarbons.

Summarised, as the hop essential oil concentration is increased, the volatile profile of boiled hop essential oil differs more from unboiled hop essential oil. In particular formation of OSs (important for the 'spicy/herbal' character of 'kettle hop' aroma) by chemical oxidation of SHCs can be positively influenced through boiling of high hop oil concentrations.

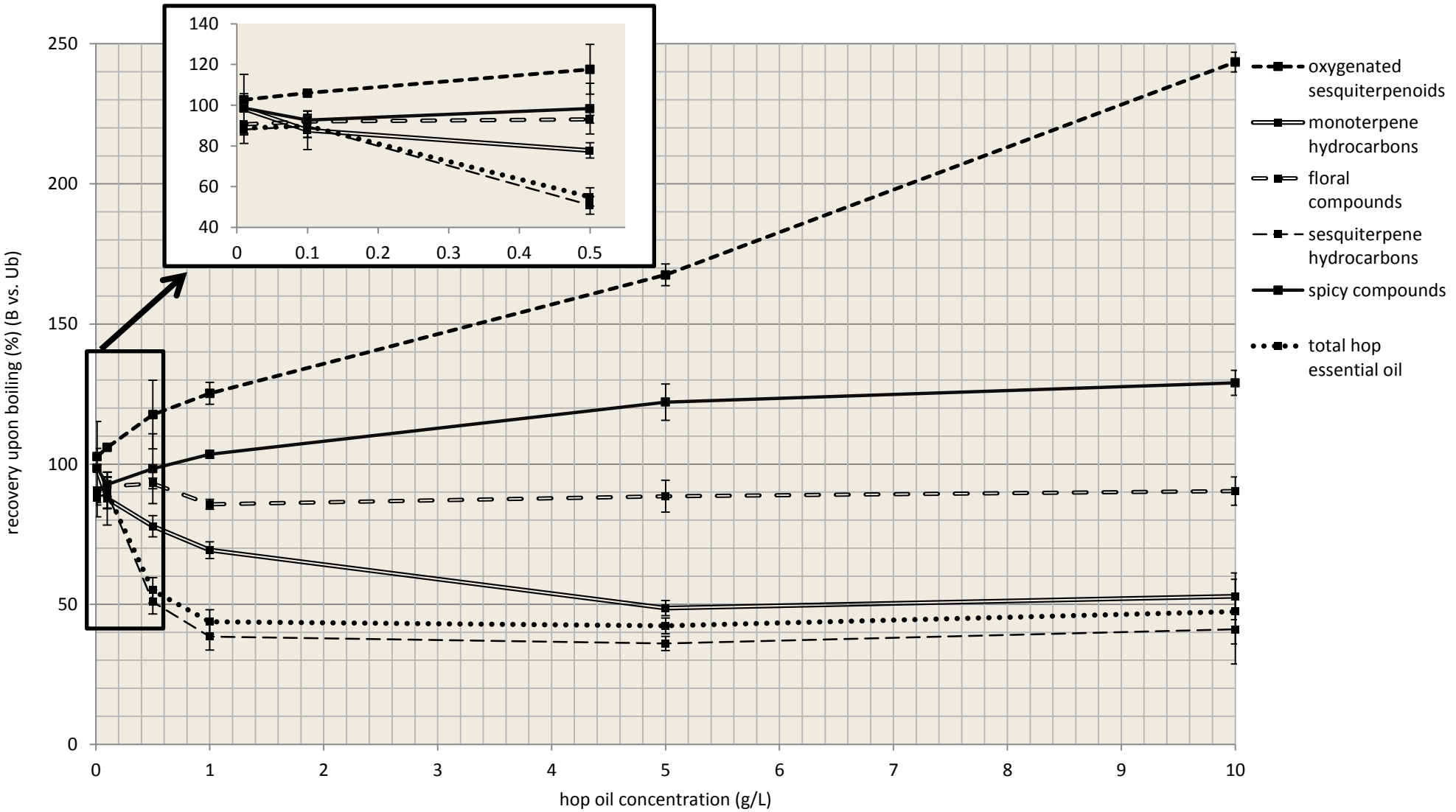

Figure 5-1. Recovery (%) of compound classes as a function of the boiled hop essential oil concentration (g/L) in MQ-water. Recoveries are calculated on the basis of the average normalised peak areas (n=2) in HS-SPME-GC-MS derived chromatograms of samples (n=3) with boiled hop essential oil (B) relative to a sample with an identical concentration of unboiled hop essential oil (U_u). Standard errors represent the standard deviation between boiled samples (n=3).

Differentiation among unboiled and boiled hop essential oil samples was explored using cluster analysis (CA). The resulting dendrogram is depicted in **Figure 5-2**, demonstrating the presence of two distinct clusters. One cluster comprises all samples originating from hop essential oil boiled in concentrations of 0.5 g/L or higher. On the other hand, boiled samples with lower hop oil concentrations are clustered jointly with unboiled hop oil samples. These observations confirm that boiling of low concentrations of hop essential oil does not lead to significant quantitative differences of the compound classes compared to the unboiled hop oil, whereas boiling of high hop oil concentrations clearly differentiates the hop-derived volatile profile from unboiled hop essential oil.

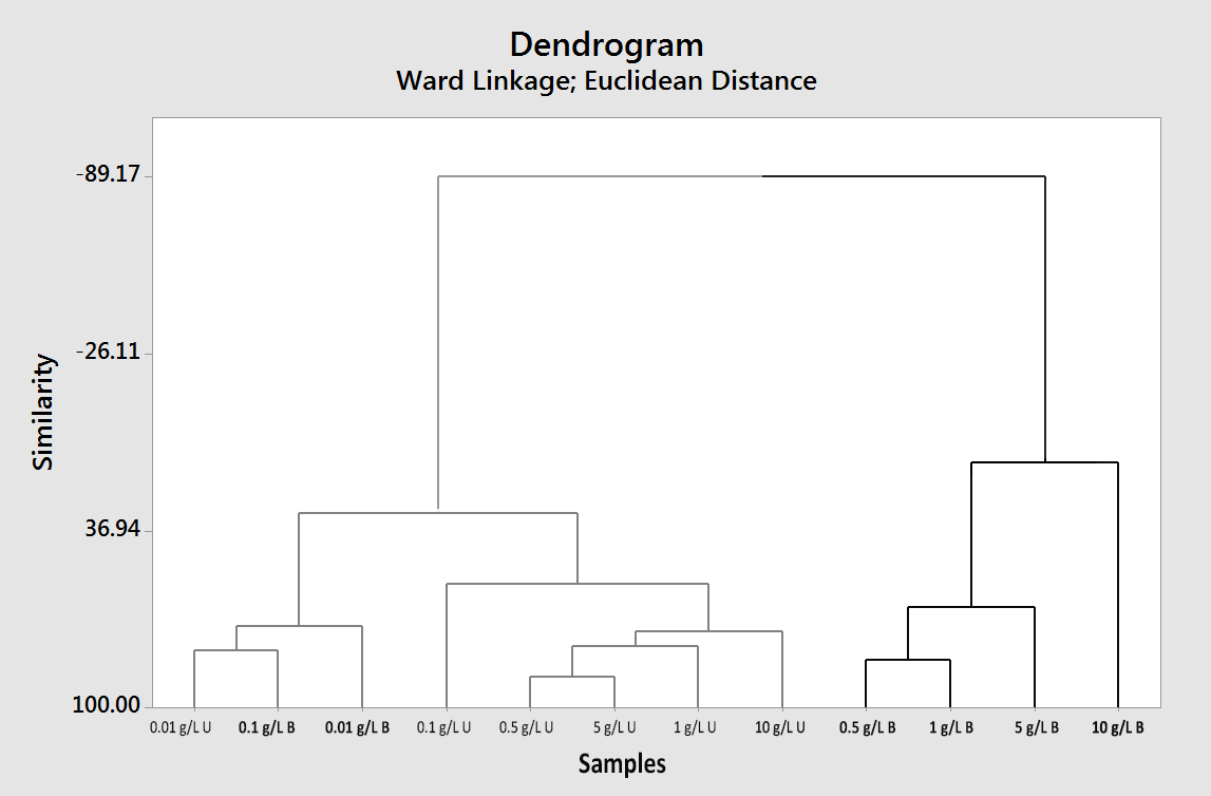

Figure 5-2. Cluster analysis on unboiled and boiled hop essential oil samples (cv. Saaz). The dendrogram was obtained by hierarchical agglomerative clustering with Ward's method. Data= average normalised peak areas; variables= compound classes (monoterpene hydrocarbons, floral fraction, sesquiterpene hydrocarbons, spicy fraction, total hop essential oil, oxygenated sesquiterpenoids); samples= unboiled hop essential oil samples (U) and boiled hop essential oil samples (B) (0.01 g/L; 0.1 g/L; 0.5 g/L; 1 g/L; 5 g/L; 10 g/L).

5.3.1.2 Boiling of hop essential oil (cv. Saaz) in sweet wort

In order to verify whether the above findings apply when boiling hop essential oil in a wort matrix, the experiment was repeated in wort. An additional reference (U_b) was used to investigate the impact of adsorption of hop oil volatiles to trub (formed during boiling of wort), which should manifest itself by a difference between the recovery calculated on the basis of U_u and U_b. Two-way ANOVA-analyses were performed separately for each

compound class in order to investigate differences between average recoveries due to the reference and to the hop oil concentration. The results (P-values to indicate whether it concerns a statistically significant difference or not, F-values to indicate magnitude of variation) are summarised in **Table 5-2 A**. It can be concluded that the hop oil concentration will clearly influence the recovery of a compound class. In particular, the hop oil concentration impacts oxidation of SHCs, since the largest F-values were observed for SHCs, total hop oil (which mainly consists of SHCs), OSs and spicy compounds (which mainly consist of OSs). Apparently, the impact of the reference seems of less importance. Although the P-values of the floral fraction and the OSs suggest a significant difference between the recovery calculated on the basis of U_u or U_b, F-values are relatively small compared to the F-values observed for the factor 'concentration'.

Table 5-2. Two way analysis of variance (ANOVA) on recoveries (n=3) of compound classes (monoterpene hydrocarbons, floral fraction, sesquiterpene hydrocarbons, spicy fraction, total hop essential oil, oxygenated sesquiterpenoids) upon boiling of total hop essential oil in water or wort at different concentrations. Confidence level= 95% (α-level= 0.05). *= statistically significant difference (P-value < 0.05). **(A)** P- and F-values for recoveries of the compound classes upon boiling in wort, factor A= the reference (U_u or U_b) on which the recovery was calculated, factor B= the boiled hop essential oil concentration. **(B)** P- and F-values for recoveries of the compound classes, calculated on the basis of the Ub reference, factor A= matrix (water or wort), factor B= the boiled hop essential oil concentration.

(A)	**Factor A= reference (U_u vs. U_b)**		**Factor B= hop oil concentration**		**interaction AB**	
Chemical compound class	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**	**F-value**
Monoterpene hydrocarbons	0.833	0.05	0.000*	18.03*	0.000*	11.27*
Floral fraction	0.025*	5.68*	0.000*	14.42*	0.000*	8.18*
Sesquiterpene hydrocarbons	0.087	3.18	0.000*	315.98*	0.000*	7.23*
Spicy fraction	0.171	2.00	0.000*	92.66*	0.005*	4.57*
Total hop essential oil	0.084	3.25	0.000*	245.65*	0.000*	9.55*
Oxygenated sesquiterpenoids	0.007*	8.81*	0.000*	422.42*	0.142	1.84
(B)	**Factor A= matrix (water vs. wort)**		**Factor B= hop oil concentration**		**interaction AB**	
Chemical compound class	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**	**F-value**
Monoterpene hydrocarbons	0.001*	15.14*	0.000*	10.00*	0.000*	16.67*
Floral fraction	0.554	0.36	0.001*	6.30*	0.237	1.47
Sesquiterpene hydrocarbons	0.000*	39.15*	0.000*	111.63*	0.002*	5.38*
Spicy fraction	0.001*	13.13*	0.000*	63.63*	0.001*	6.31*
Total hop essential oil	0.000*	34.48*	0.000*	91.16*	0.001*	5.63*
Oxygenated sesquiterpenoids	0.000*	20.03*	0.000*	421.25*	0.000*	18.42*

Since use of the latter reference gives a more realistic image of quantitative changes upon boiling, recoveries in **Figure 5-3** were alculated on the basis of the U_b sample. The graphs for the different compound classes show similar behaviour to the graphs obtained upon boiling of hop essential oil in MQ-water (see), suggesting that oxidation of SHCs into oxygenated derivatives exhibits comparable concentration-dependency when boiling hop oil in sweet wort.

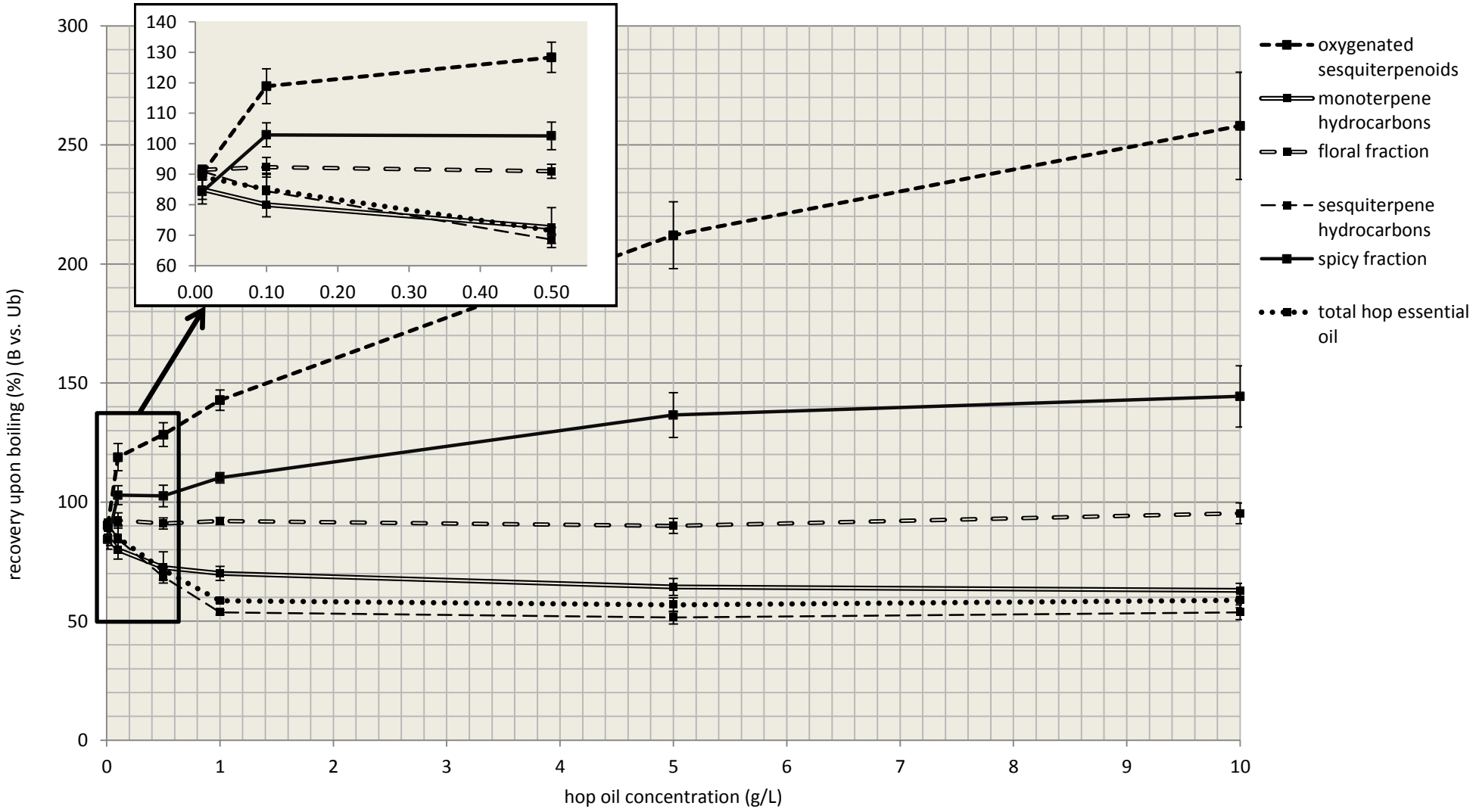

Figure 5-3. Recovery (%) of compound classes as a function of the boiled hop essential oil concentration (g/L) in wort. Recoveries are calculated on the basis of the average normalised peak areas (duplicate analysis) in HS-SPME-GC-MS derived chromatograms of samples (n=3) with boiled hop essential oil (B) relative to a sample with an identical concentration of unboiled hop essential oil (U_b). Standard errors represent the standard deviation between boiled samples (n=3).

Potential recovery differences upon boiling due to the matrix (water vs. sweet wort) and hop oil concentration were investigated into more detail using two-way ANOVA analysis for each compound class (see **Table 5-2 B**). Once more, differences in hop oil concentration induce significant recovery differences and the largest F-values are again observed for SHCs, total hop oil, spicy compounds and, in particular, OSs. P-values for the factor 'matrix' (water or wort) indicate statistically significant differences in recovery for all compound classes, except for the floral compounds. Variation due to the matrix is much smaller than variation due to the hop oil concentration (except for monoterpene hydrocarbons). However, there can be concluded that also the matrix in which hop oil is boiled has an impact on recoveries of different compound classes. Remarkably, OSs show significantly higher recoveries in wort compared to water (see **Figure 5-3** vs**.**), which might imply higher reactivity of SHCs in the complex wort matrix. From **Table 5-2** there can also be concluded that the recovery significantly differs for references and matrices depending on the hop oil concentration, except for OSs and floral compounds, respectively.

5.3.2 Investigation of varietal differences in the analytical fingerprint of hop-derived volatiles upon boiling of hop essential oil in sweet wort (cv. Saaz, Hallertau Tradition, Perle and Magnum)

5.3.2.1 Principal component analysis (PCA) for screening group structures in unboiled and boiled varietal hop essential oil dilutions in wort

In order to reveal changes of individual hop oil volatiles upon lab-scale boiling of hop essential oil (cv. Saaz) in wort, the volatile profile of boiled hop essential oil (10 g/L) was compared to unboiled hop oil by comprehensive analytical characterisation. Varietal differences were investigated by repeating the experiment with two other aroma hop varieties (cv. Hallertau Tradition and Perle) and one high alpha hop (cv. Magnum). Information on the hop varieties used can be found in **Table 5-3**.

In the first instance, the data derived from the different hop varieties was screened for group structures using PCA. The biplot, given in **Figure 5-4**, shows a clear intra-varietal distinction between unboiled (U_b) and boiled (B) hop essential oil, as well as inter-varietal differences. Unboiled hop oils tend to be characterised by higher terpene levels compared to their boiled counterparts, whereas the scores of boiled hop oil samples lie closer to the loadings of the oxygenated compounds. Upon boiling, a similar shift of the samples derived from the aroma hop varieties can be observed in the PCA biplot. The unboiled samples from the noble varieties are characterised by higher SHC levels, whereas the unboiled samples from the bitter variety cv. Magnum are distinguished by higher monoterpene levels

(especially β-myrcene). Of the unboiled hop essential oils, the samples from cv. Saaz are closest to the loadings of oxygenated compounds, which might be due to the poor storage stability of this particular variety[103]. In contrast, of the aroma hop varieties, unboiled hop oils cv. Perle are located the furthest from oxygenated compounds, which might be explained by the excellent storage stability of cv. Perle[103,104]. Hop essential oil samples cv. Magnum contain relatively low levels of floral and spicy compounds, even upon boiling. This observation is in agreement with the work of Foster[104], who found that hops characterised by low total hoppiness potential (sum of floral compounds, citrus compounds and oxidation products), both in fresh and aged hop samples, usually show good storage stability.

Table 5-3. Characteristics of hops cv. Magnum, Hallertau Tradition, Perle and Saaz ('Barth-Haas Hops Companion'[103]).

Variety	Hallertau Magnum	Hallertau Tradition	Hallertau Perle	Saaz
Origin	Germany	Germany	Germany	Czech Republic
Hop type	High α hop	Aroma hop	Aroma hop	Aroma hop
Pedigree	Daughter of US Galena	Daughter of Hallertau Gold	Bred from Northern Brewer	Czech landrace variety
Aroma	Spicy with some fruity note	Noble-type with fruity notes	Herbal, spicy	Classic noble aroma (associated with classic pilsner beer)
α-acids (w/w%)	11.0-16.0 %	4.0-7.0 %	4.0-9.0 %	3.0-6.0 %
β-acids (w/w%)	5-7 %	3.0-6.0 %	2.5-4.5 %	4.5-8.0 %
Cohumulone	21-29 % of α-acids	24-30 % of α-acids	29-35 % of α-acids	23-26 % of α-acids
Total oil	1.6-2.6 mL/100g	0.5-1.0 mL/100g	0.5-1.5 mL/100g	0.4-1.0 mL/100g
Myrcene	30-45 % of total oil	14-32 % of total oil	20-35 % of total oil	25-40 % of total oil
Humulene	30-45 % of total oil	35-50 % of total oil	35-55 % of total oil	15-25 % of total oil
Caryophyllene	8-12 % of total oil	10-15 % of total oil	10-20 % of total oil	10-12 % of total oil
Farnesene	<1 % of total oil	<1 % of total oil	<1 % of total oil	14-20 % of total oil
Storage stability	Very good	Good	Very good to excellent	Very poor to poor

5.3.2.2 Composition of unboiled varietal hop essential oil dilutions in wort

The composition of varietal unboiled hop oil dilutions in wort (cv. Magnum, Hallertau Tradition, Perle, Saaz) is depicted in **Figure 5-5**. Clearly, the aroma hop varieties investigated in this work contain higher sesquiterpene levels, in particular α-humulene, whereas cv. Magnum appears to be rich in monoterpene hydrocarbons (≥ 90% β-myrcene), as also reported in literature[27,28,105,106].

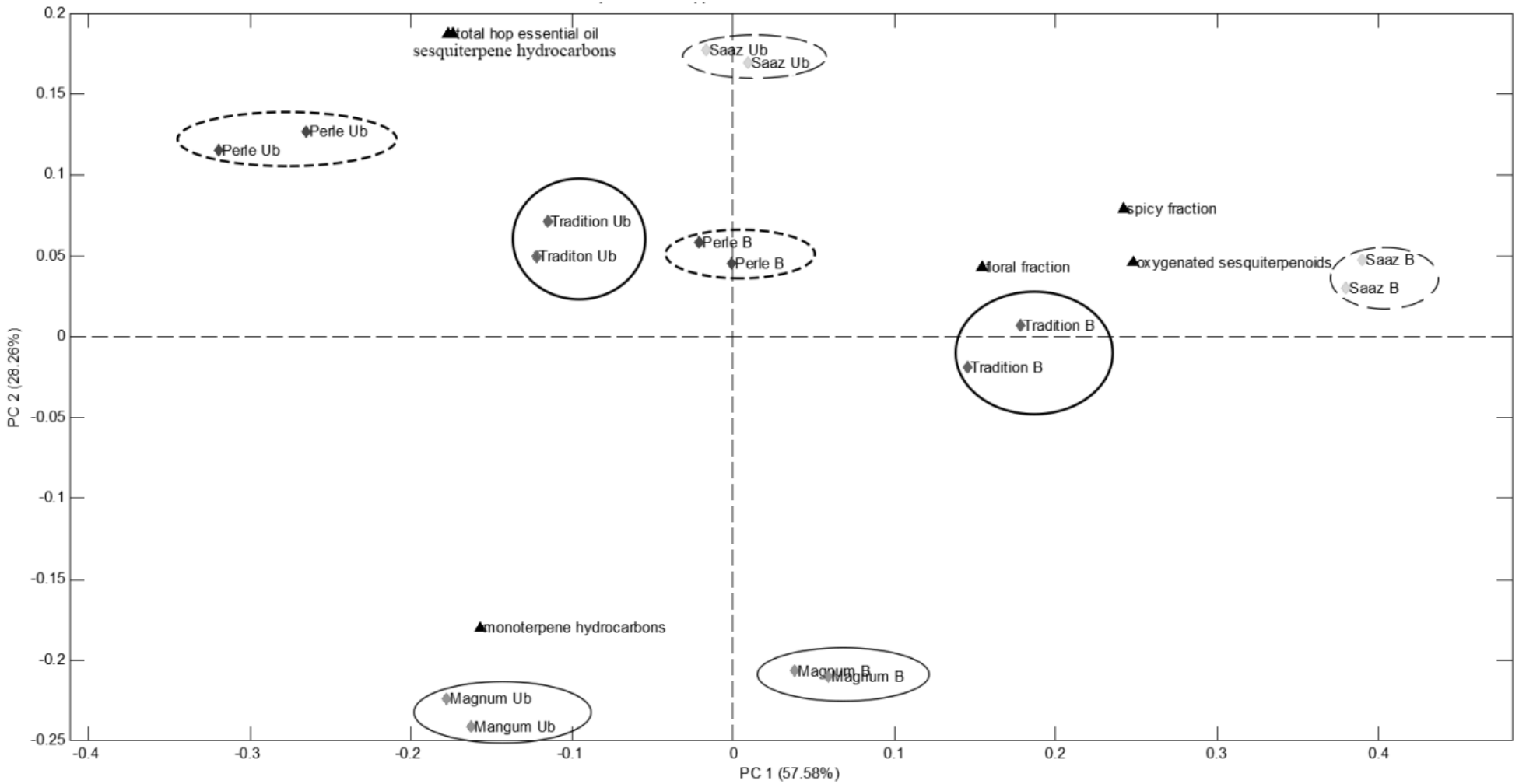

Figure 5-4. PCA biplot of normalised peak areas of different compound classes, detected in chromatograms of unboiled and boiled varietal hop essential oil (cv. Magnum, Hallertau Tradition, Perle and Saaz) in wort. Samples: unboiled (U_b) and boiled (B) samples; variables: compound classes. PCA preprocessing via autoscale; selection of 2 PCs explaining 85.8 % of variance.

When focusing on the composition of the spicy fraction, one can see that the levels of α-humulene and β-caryophyllene oxidation products, selinene/cadinene-derived alcohols as well as miscellaneous compounds (e.g. alcohols, esters, ketones such as 6Z-pentadecen-2-one and 2-pentadecanone, and several SHCs eluting in the spicy region) are the lowest in hop oil cv. Magnum. According to Peacock *et al.*[18], concentrations of cadinols and α-eudesmol tend to be higher in noble aroma hops and, indeed, the unboiled hop oils derived from the aroma varieties investigated by us contain almost two times more selinene/cadinene-derived alcohols than hop oil cv. Magnum. Unboiled hop oil cv. Saaz clearly shows the highest level of α-humulene and β-caryophyllene oxidation products, which might be the consequence of its storage instability[103]. Therefore, brewers desiring hops with high levels of oxidation products and thus 'hoppiness' potential, may select hops with high levels of α-humulene and β-caryophyllene and consider (mild) ageing of these hop varieties before brewing[104].

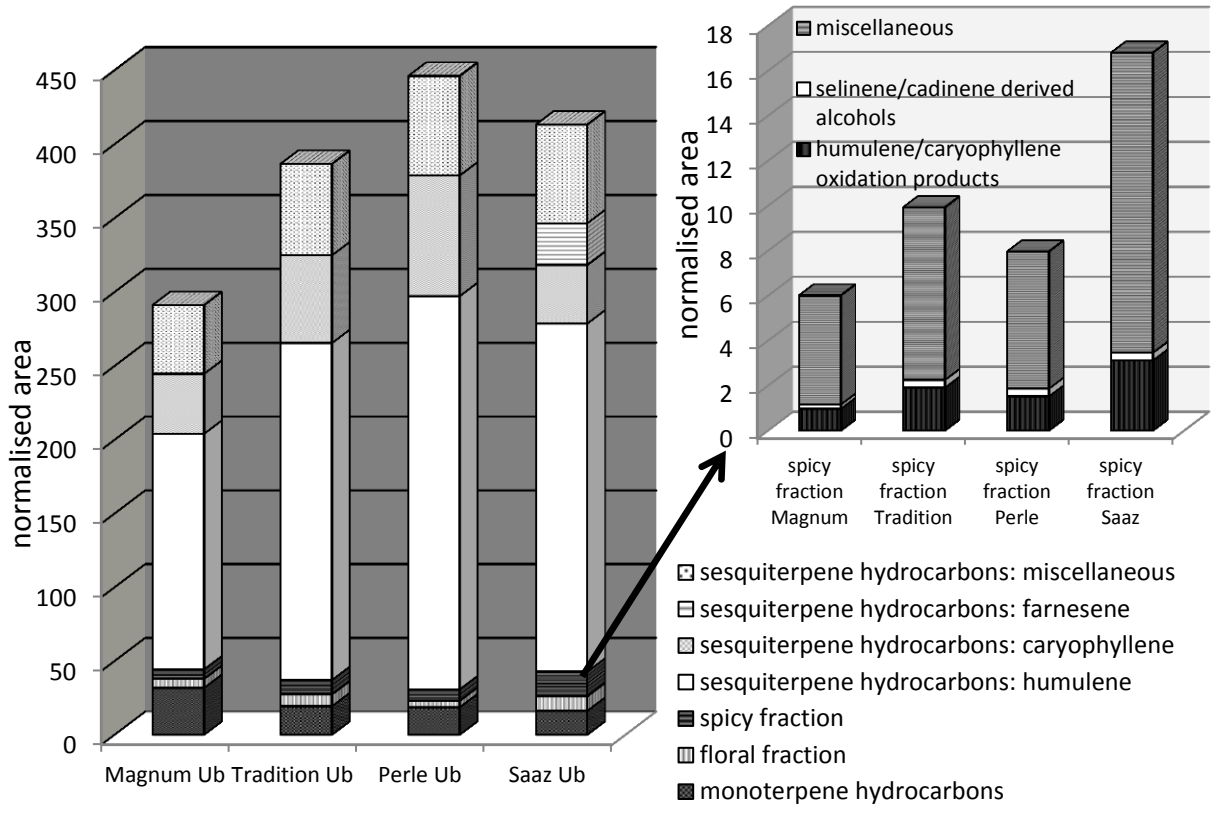

Figure 5-5. Normalised peak areas of the spicy, floral, sesquiterpene and monoterpene hydrocarbon fractions of unboiled hop oil dilutions (cv. Magnum, Hallertau Tradition, Perle, Saaz) in wort. The spicy fraction is further subdivided into humulene and caryophyllene oxidation products, selinene and cadinene-derived alcohols and miscellaneous compounds (non-oxygenated sesquiterpenoids, *e.g.* aliphatic alcohols, ketones, esters etc.).

5.3.2.3 Recoveries of individual volatiles upon boiling of varietal hop essential oil dilutions in wort.

The relative peak area (%) and the recovery (%) upon boiling were calculated for each hop-derived volatile detected in the profile of unboiled (U_b) and boiled (B) samples (cv. Magnum, Perle, Hallertau Tradition and Saaz). **Table 5-4** shows these data for all compounds which proved to increase in their level upon boiling (recovery > 100%) and for compounds detected in the boiled hop essential oil sample but not in the corresponding unboiled hop oil sample (*i.e*. newly formed upon boiling). Apparently, largely independent of the hop variety, chemically identical compounds are found to increase in their level upon boiling. Exceptions are an unknown sesquiterpene hydrocarbon (only detected in hop oils cv. Hallertau Tradition and Magnum) and 3 oxygenated sesquiterpenoids (only detected in hop oils cv. Saaz); *i.e*. an unknown oxygenated sesquiterpenoid, 14-hydroxy-β-caryophyllene, and E-dendrolasin (β-farnesene-derivative, also present in cv. Sterling[210]). 14-Hydroxy-β-caryophyllene is an odour impact compound ('cedarwood') of spicy fractions cv. Cascade, Target, Hallertauer Hersbrucker and Saaz[114] and we also detected this compound in an odour-active region upon GC-O analysis of a kettle hopped lager beer (**Chapter 3**). Dendrolasin was only detected in hop essential oil cv. Saaz, which is well-known for its relatively high β-farnesene levels[80,103]. Guilico and coworkers[219] suggested a possible derivation of the furan sesquiterpene dendrolasin from its corresponding acyclic member (farnesol/farnesal). Analogously, in the C_{10} series, the furan monoterpene perillene is structurally related to citral (or geraniol)[219].

Although most volatiles found in **Table 5-4** are oxygenated compounds, particular terpenes are also detected amongst hop-derived volatiles characterised by an increase upon boiling. P-cymene, *trans*-calamenene, β-calacorene, α-corocalene and cadalene are such examples. P-cymene, was already found decades ago upon auto-oxidation of β-myrcene by Dieckmann and Palamand[136]. These authors proposed a disproportionation reaction under influence of oxygen and heat, involving 2 molecules of limonene and yielding 1 molecule of 8(9)-menthadiene and 1 molecule of p-cymene. A similar reaction might explain the elevated levels of p-cymene detected by us upon boiling hop oil. Interestingly, the sesquiterpene hydrocarbons characterised by an increase in their level upon boiling have additional unsaturated bonds in common (calamenene: $C_{15}H_{22}$, β-calacorene and α-corocalene: $C_{15}H_{20}$, cadalene: $C_{15}H_{18}$, compared to the typical empirical formula $C_{15}H_{24}$), suggesting that they were formed through oxidation. In **Chapter 2**, we also found elevated cadalene levels after boiling. According to Bülow and König[220], cadalene, α- and γ-calacorene as well as *cis/trans*-calamenene were found amongst oxidation products formed from γ-muurolene. Although we did not detect these calacorenes, we observed an increase in the level of β-calacorene (an isomer) upon boiling.

Table 5-4. Volatile compounds, showing an increase in their level upon boiling (recovery B vs U_b > 100% or detected in B but not in U_b) of hop essential oil (cv. Magnum, Perle, Hallertau Tradition and Saaz) in wort at a concentration of 10,000 mg/L. RI= calculated Retention index. IDENT.= method of identification, on the basis of RC (reference compound), RI (Retention index), MS (mass spectrum) and, comparison of MS and RI of compounds with MS and RI of compounds in reference mixtures (codes: c= caryophyllene, h= humulene, ic= isocaryophyllene, co= caryophyllene oxide, ho= humulene epoxide, EP= epoxidation, HP= hydrolysis product/ acid-catalysed rearrangement, PO= photosensitised oxidation). U%= average relative peak area in U_b. B%= average relative area in B. stdev= standard deviation of relative area (n=2). R= % recoveries of volatiles calculated on basis of normalised peak areas in boiled (B) hop oil samples to peak areas in unboiled hop oils (U_b). N= newly formed upon boiling (not detected in U_b). n.d.= not detected.

			MAGNUM					PERLE					TRADITION					SAAZ				
COMPOUND	**RI**	**IDENT.**	**U%**	**stdev**	**B%**	**stdev**	**R**	**U%**	**stdev**	**B%**	**stdev**	**R**	**U%**	**stdev**	**B%**	**stdev**	**R**	**U%**	**stdev**	**B%**	**stdev**	**R**
p-Cymene	1013	RC, MS, RI	0.000	0.000	0.017	0.001	N	0.007	0.000	0.014	0.000	148	0.000	0.000	0.018	0.000	N	0.007	0.000	0.014	0.001	122
Perillene	1088	MS, RI	0.017	0.000	0.279	0.003	1298	0.007	0.001	0.081	0.000	865	0.028	0.001	0.187	0.003	472	0.031	0.000	0.211	0.004	417
Unknown sesquiterpene hydrocarbon	1427	-	0.043	0.008	0.100	0.005	181	n.d.		n.d.			0.080	0.002	0.215	0.031	190	n.d.		n.d.		
Unknown oxygenated sesquiterpenoid (m/z 69, 81, 95, 109, 123, 138, 149, 205, 220)	1469	-	0.000	0.000	0.208	0.000	N	0.000	0.000	0.129	0.009	N	0.000	0.000	0.229	0.017	N	0.000	0.000	0.153	0.000	N
trans-Calamenene	1502	MS, RI	0.198	0.009	0.948	0.021	371	0.304	0.007	1.208	0.008	300	0.137	0.003	0.504	0.004	261	0.480	0.009	1.185	0.002	149
(4S)-Dihydrocaryophyllene-5-one	1525	MS, RI	0.000	0.000	0.037	0.001	N	0.000	0.000	0.021	0.002	N	0.000	0.000	0.026	0.000	N	0.000	0.000	0.000	0.000	N
Isocaryophyllene epoxide A	1526	icEP, MS, RI	0.000	0.000	0.015	0.002	N	0.000	0.000	0.034	0.004	N	0.000	0.000	0.040	0.004	N	0.000	0.000	0.012	0.001	N
(4R)-Dihydrocaryophyllene-5-one	1528	MS, RI	0.000	0.000	0.013	0.001	N	0.000	0.000	0.015	0.003	N	0.000	0.000	0.011	0.002	N	0.000	0.000	0.011	0.001	N
6(5→4)-Abeo-caryophyll-7-en-5-al	1532	MS, RI	0.000	0.000	0.004	0.000	N	0.000	0.000	0.010	0.001	N	0.000	0.000	0.009	0.003	N	0.000	0.000	0.011	0.004	N
β-Calacorene	1535	MS, RI	0.002	0.000	0.008	0.001	234	0.003	0.000	0.010	0.000	221	0.004	0.000	0.013	0.000	219	0.004	0.000	0.011	0.001	182
Unknown oxygenated sesquiterpenoid (m/z 93, 205, 220)	1542	-	n.d.		n.d.			n.d.		n.d.			n.d.		n.d.			0.007	0.000	0.037	0.005	322
Humuladienone	1544	MS, RI	0.000	0.000	0.135	0.000	N	0.005	0.000	0.033	0.003	528	0.008	0.000	0.050	0.004	452	0.023	0.001	0.067	0.001	179
6(5→4)-Abeo-caryophyll-8(13)-en-5-al	1550	MS, RI	0.019	0.001	0.222	0.005	900	0.027	0.001	0.241	0.015	675	0.038	0.002	0.233	0.012	433	0.009	0.000	0.041	0.003	281
E-Dendrolasin	1552	MS, RI	n.d.		n.d.			n.d.		n.d.			n.d.		n.d.			0.029	0.000	0.397	0.006	819
Caryophyllene oxide	1553	cEP, RC, MS, RI	0.016	0.001	0.039	0.005	188	0.012	0.002	0.051	0.002	328	0.021	0.005	0.071	0.001	240	0.002	0.000	0.026	0.003	659
Clovenol	1555	coHP, MS, RI	0.010	0.006	0.036	0.002	271	0.006	0.002	0.052	0.006	652	0.012	0.001	0.068	0.008	414	0.002	0.000	0.009	0.000	348
Humulene epoxide I	1568	hEP, MS, RI	0.012	0.001	0.122	0.002	823	0.015	0.003	0.115	0.002	562	0.028	0.004	0.179	0.003	460	0.045	0.003	0.209	0.008	281
Humulene epoxide II	1578	hEP, MS, RI	0.058	0.004	0.307	0.008	410	0.024	0.007	0.274	0.002	857	0.046	0.010	0.424	0.002	656	0.092	0.007	0.507	0.048	335
Humulene allylic alcohol	1588	hPO, MS, RI	0.008	0.003	0.022	0.003	217	0.000	0.000	0.029	0.001	N	0.012	0.001	0.028	0.003	169	0.000	0.000	0.000	0.000	625
α-Corocalene	1592	MS, RI	0.009	0.001	0.025	0.001	207	0.002	0.001	0.009	0.000	281	0.015	0.002	0.047	0.005	229	0.003	0.000	0.009	0.000	155
Humulene epoxide III	1599	hEP, MS, RI	0.024	0.003	0.328	0.007	1069	0.022	0.003	0.299	0.014	1019	0.042	0.005	0.437	0.012	740	0.047	0.002	0.386	0.021	496
Humulenol II	1601	hPO, MS, RI	0.047	0.002	0.654	0.008	1084	0.074	0.004	0.554	0.020	567	0.107	0.007	0.812	0.044	539	0.145	0.003	0.835	0.053	348
Caryophylla-4(12),8(13)-diene-5-ol	1603	cPO, MS, RI	0.000	0.000	0.184	0.002	N	0.000	0.000	0.143	0.001	N	0.010	0.000	0.198	0.014	1371	0.040	0.002	0.201	0.013	304
(3Z)-Caryophylla-3,8(13)-diene-5α-ol	1613	cPO, MS, RI	0.000	0.000	0.240	0.002	N	0.012	0.002	0.251	0.010	1523	0.022	0.004	0.314	0.011	1019	0.028	0.003	0.295	0.008	641
14-Hydroxy-β-caryophyllene	1637	MS, RI	n.d.		n.d.			n.d.		n.d.			n.d.		n.d.			0.056	0.003	0.116	0.021	125
(3Z)-Caryophylla-3,8(13)-diene-5β-ol	1620	cPO, MS, RI	0.051	0.010	0.177	0.000	272	0.038	0.005	0.135	0.004	270	0.037	0.003	0.182	0.013	350	0.030	0.001	0.194	0.027	390
Cadalene	1620	MS, RI	0.007	0.007	0.014	0.000	153	0.002	0.003	0.017	0.001	530	0.004	0.000	0.027	0.001	526	0.004	0.000	0.024	0.003	328
Humulene allylic alcohol	1624	hPO, MS, RI	0.145	0.008	0.279	0.003	149	0.000	0.000	0.185	0.004	N	0.095	0.001	0.278	0.004	208	0.072	0.004	0.441	0.047	369

In addition, *trans*-calamenene was also characterised by an increase upon boiling and this compound is known as a direct oxidation product of cadina-3,5-diene[221]. The above observations provide support for conversion of SHCs into other SHCs, more precisely via elimination reactions (with formation of a double bond) under aerobic conditions and at elevated temperatures.

As mentioned earlier (**Chapter 2** and **4**), we did not detect an increase in the level of cadinols, eudesmols and cubenols upon boiling. Literature data suggests that cadinols might not be chemical oxidation products but are instead biosynthesised by the hop plant[18]. On the other hand, Tressl and coworkers[78] synthesised epoxidation products of δ-cadinene, which were subsequently reduced using $LiAlH_4$, yielding δ-cadinol, τ-muurolol, *epi*-cubenol and cubenol. Reduction of β-selinene epoxides yielded selinen-11-en-4-ol and β-eudesmol. In analogous reactions α-cadinol and τ-cadinol were formed from the epoxides of γ-cadinene and selin-7-en-4-ol (juniper camphor) from the epoxide of selina-4,7-diene. The authors detected these bicyclic alcohols (except for τ-muurolol) in Hersbrucker Spät hops[78]. During a 3-year long storage of Spalter hops, *epi*-cubenol, δ-cadinol, τ-cadinol and α-cadinol proved to slightly increase in their level[82]. These increases were however not comparable with the much higher increases observed for α-humulene and β-caryophyllene oxidation products.

To further investigate if cadinenes and selinenes might be oxidised into the bicyclic sesquiterpene alcohols discussed above, and, because SHCs are not that easily oxidised during boiling in an aqueous matrix (they remain largely untransformed[17], see also **Chapter 2** and **4**), we selected a hop variety rich in selinenes and cadinenes. Levels of selinenes and cadinenes in hop oils are in general much lower than those of α-humulene and β-caryophyllene, except for e.g. Pride of Ringwood[80], which contains 2% α-humulene, 12% β-caryophyllene, 11% β-selinene and 13% α-selinene[222]. Also Super Pride is characterised by relatively high selinene levels (i.e. 17% β-selinene and 19% α-selinene compared to 1% α-humulene and 6% β-caryophyllene)[222]. Consequently, we selected cv. Super Pride and isolated its SHC fraction via SPE (as described in **Chapter 4**). The resulting fraction contained 4.85 ± 0.19 % monoterpene hydrocarbons, 0.15 ± 0.03 % oxidation products and 95.00 ± 0.20 % SHCs (n=5) (results not shown). More specifically, the fraction contained only 10.13 ± 0.16 % β-caryophyllene and 2.32 ± 0.02% α-humulene, whereas β-selinene, α-selinene, selina-4,11-diene, 7-epi-α-selinene and selina-3,7(11)-diene make up respectively 33.24 ± 0.07 %, 33.15 ± 0.06 %, 6.16 ± 0.03 %, 1.00 ± 0.01 % and 0.07 ± 0.00% of the total fraction. Relative percentages (on the basis of relative peak areas) of δ-cadinene, γ-cadinene, α-cadinene and trans-cadina-1,4-diene proved much lower; respectively 1.36 ± 0.02 %, 0.98 ± 0.01 %, 0.23 ± 0.00 % and 0.18 ± 0.00 % of the total fraction.

The sesquiterpene hydrocarbon fraction cv. Super Pride was boiled on a lab scale (as described in **Chapter 4**) and, although a large series of unidentified oxygenated sesquiterpenoids was formed *de novo* upon boiling of the SHC fraction (the relative percentage of oxidation products increased to 5.47 ± 0.59 % in the boiled fraction), the cubenols, cadinols, muurolols, eudesmols and selinenols discussed above were not detected amongst these compounds. Apparently these sesquiterpene alcohols are not detected upon boiling of a selinene-rich SHC fraction cv. Super Pride under our applied conditions.

5.3.3 GC-olfactometry for determination of flavour-active compounds upon boiling of hop essential oil (cv. Saaz) in wort

Hop essential oil (cv. Saaz) samples boiled in wort were diluted with water and subjected to GC-olfactometry to determine flavour-active compounds. Samples derived from the highest initial hop oil concentration (10 g/L) were chosen for these assessments since these samples contain the highest levels of oxidation products formed *de novo* upon boiling. **Figure 5-6** depicts the clearest flavour-active zones (*i.e.* detected 4 times or more out of the 9 analyses) and the compounds eluting within these zones. Among the compounds characterised by an increase in their level upon boiling (see **Table 5-4**), perillene, *trans*-calamenene, cadalene, and a series of sesquiterpene oxidation products (*i.e.* E-dendrolasin, 6(5→4)-abeo-caryophyll-8(13)-en-5-al, caryophyllene epoxide, humulene epoxide III, humulenol II, caryophylla-4(12),8(13)-diene-5-ol, (3Z)-caryophylla-3,8(13)-diene-5α/β-ol and 14-hydroxy-β-caryophyllene) were also detected in flavour-active intervals (see **Figure 5-6**). Humulene epoxide III, humulenol II, caryophylla-4(12),8(13)-diene-5-ol, (3Z)-caryophylla-3,8(13)-diene-5α/β-ol and 14-hydroxy-β-caryophyllene were previously found in flavour-active zones of an OS hop fraction, which imparted 'spicy/woody/hoppy' notes when added to an iso-α-acid bittered lager (**Chapter 4**). Moreover, the same compounds were also detected in flavour-active zones of spicy fractions derived from a commercial lager, exclusively kettle hopped with German noble aroma hop varieties (cv. Hallertau Mittelfrüh and cv. Tettnang Tettnanger) (**Chapter 3**). Our current findings are clearly in agreement with these previous independent studies, proving that our earlier results obtained upon boiling in water also apply when boiling in wort, and thus pointing to the relevance of the boiling process for the development of 'kettle hop' aroma.

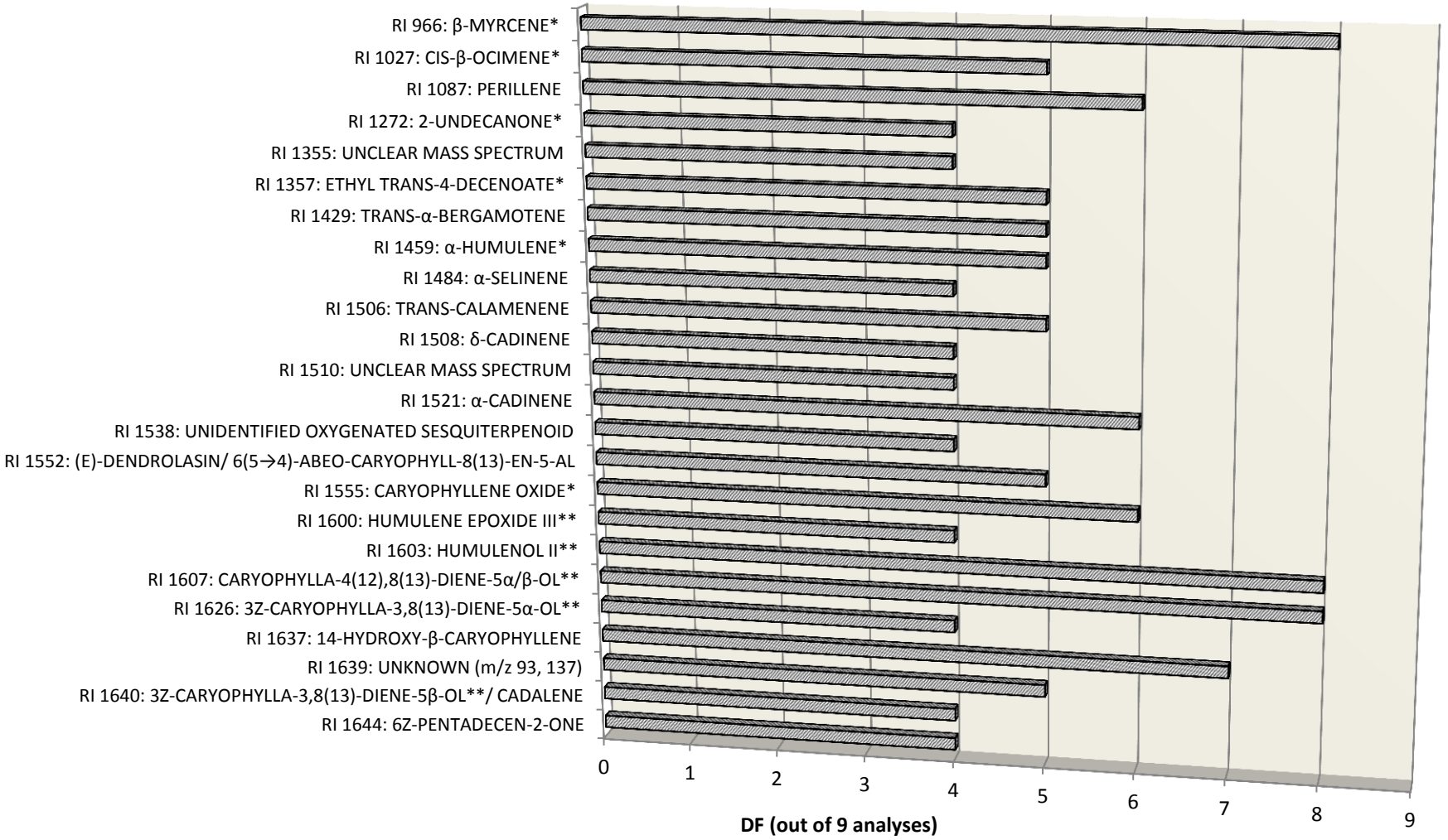

Figure 5-6. Detection frequency (DF) of flavour-active zones and compounds eluting in these zones upon GC-O analysis of boiled hop essential oil (cv. Saaz; 10,000 mg/L) in wort. (Tentative) identification of volatiles on the basis of mass spectrum (MS) and retention index (RI), *= on the basis of MS, RI and reference compound, **= on the basis of MS, RI and mixture of reference compounds.

5.4 Conclusions

For the first time, we proved a positive correlation between the initial hop oil concentration and formation of OSs upon boiling, which might find interesting applications in brewing practice. Moreover, quantitative changes in hop oil compound classes upon boiling of hop essential oil in water and wort are highly comparable, pointing to the relevance of our previous lab scale boiling experiments in water for real brewing.

In brewing practice, noble European aroma hops may be added at an early stage of kettle boiling if a subtle yet distinct 'noble kettle hop' aroma is desired in the final beer. The 'spicy' and 'herbal' notes that characterise this type of hop-derived aroma are associated with the presence of OSs and, in the previous chapter (**Chapter 4**), we proved a cause-and-effect relationship between the occurrence of SOPs in beer and 'hoppy', 'spicy' and 'woody' scents. In our current experiment, most of the volatiles formed *de novo* upon boiling of cv. Saaz also showed an increase in their level upon boiling of cv. Hallertau Tradition, Perle and Magnum. However, some minor compounds were exclusively detected upon boiling of a particular variety, indicating that differences in the qualitative spectrum of volatiles formed *de novo* upon boiling exist between different varieties. These minor differences might significantly impact the flavour profile (e.g. presence of the odour-active compound 14-hydroxy-β-caryophyllene in cv. Saaz) and the initial intrinsic hop oil composition might explain such differences (e.g. presence of E-dendrolasin in cv. Saaz upon boiling can be attributed to presence of β-farnesene in unboiled hop oil). Differences in OS levels between boiled hop oils of different varieties might also be explained by the intrinsic hop oil composition. For example, hop varieties containing high α-humulene and β-caryophyllene levels may give rise to higher SOP levels upon boiling and also initial OS levels (both SOPs formed during storage of hops and plant metabolism-related OSs) account for the total OS level in boiled hop oils. Summarised, the intrinsic hop oil composition of a particular variety might be decisive for the OS spectrum upon boiling of hop oil and might thus play a key role in the potential of hop varieties to develop 'kettle hop' aroma during the brewing process.

Upon boiling of a SHC fraction cv. Super Pride, a variety that contains high selinene levels, we could not detect *de novo* formation of selinenols and related compounds (*i.e.* cadinols, muurolols, eudesmols). Thus, our novel approach provides indirect evidence supporting the hypothesis that these compounds are biosynthesised by the hop plant.

About 20 compounds were found in flavour-active zones upon boiling of hop essential oil cv. Saaz in wort. Since some of these compounds are also formed *de novo* during the boiling process, they may play an important role into the development of 'kettle hop' aroma in real brewing practice.

Chapter 6

CHEMICAL AND SENSORIAL CHARACTERISATION OF CONVENTIONALLY AND ADVANCED HOPPED PILOT-SCALE LAGER BEERS

A part of chapter 6 corresponds to:

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Brewing of conventionally hopped beers and of beers hopped with hop essential oil-derived fractions (cv. Saaz). Chemical-analytical and sensory characterisation of worts and beers. GCxGC-TOFMS for comprehensive profiling of sesquiterpene oxidation product fraction and beer brewed with this fraction.

Tatiana Praet and Julien Smeyers (bachelor student) performed the analytical experiments. Ing. Brecht De Causmaecker performed the brewing trials. We are also grateful to Dr. Stefan Voorspoels for the opportunity to perform comprehensive two-dimensional gas chromatography mass spectrometry (GCxGC-TOFMS) at the Flemisch Institute for Technological Research (VITO, Mol, Belgium), and, to Diane Bertels and Geert Cremers for their helpful suggestions and professional assistance. The final manuscript was written by Tatiana Praet and revised and adapted after critical input by Dr. Filip Van Opstaele and Prof. Luc De Cooman.

6 CHEMICAL AND SENSORIAL CHARACTERISATION OF CONVENTIONALLY AND ADVANCED HOPPED PILOT-SCALE LAGER BEERS

6.1 Introduction

Various parameters, such as hop variety, growing region, hop product and hopping regime, have a major impact on hop flavour in beer. The point of time of hop addition is definitely decisive in this regard[20,162–164]. The impact of 'late kettle' and 'whirlpool' hopping technologies on 'hoppy' flavour is scientifically quite well understood and linalool has been proven to be an important contributor to the resulting 'floral' notes[10–12,14]. On the other hand, insights into 'early kettle' hopping and the possibly resulting 'spicy/herbal' aspect of 'kettle hop' flavour aspect of beer appear to be elusive.

It has been suggested in literature that chemical oxidations of terpene hydrocarbons occur during kettle boiling[10,16,30,39,113,134]. Several researchers have indeed found indications for such oxidation reactions[10,20,135]. Nevertheless, *de novo* formation of sesquiterpene oxidation products (SOPs) has not been unambiguously demonstrated in real brewing practice. The impact of addition of hops at the onset of wort boiling on 'kettle hop' flavour has even been questioned. Meilgaard and Peppard stated that beers resulting from this hopping practice would rarely exhibit any appreciable degree of hop character[39] and results from Kaltner and coworkers would point to the fact that oxidation products are not involved in contributing to hop aroma in beer[132,162,223]. Fritsch and Schieberle did not detect additionally formed compounds as a result of 'early' kettle hopping and stated that this finding is contradictory to the often mentioned formation of new odour-active compounds when hops are boiled[176]. Summarised, the impact of 'early kettle' hopping with regard to generation of new odourants and the 'hoppy' flavour in the final beer remains a matter of debate.

To shed light on this complex issue we have been conducting lab scale boiling experiments with total hop essential oil and sesquiterpene hydrocarbon (SHC) fractions in simplified model solutions, see **Chapters 2-5**. Various sesquiterpene oxidation products were formed *de novo* upon boiling in both water and wort. Moreover, several of these constituents were found in flavour-active zones upon GC-O and were also detected in beer. We also demonstrated that, both boiled hop essential oil and hop-derived SOPs exhibited 'spicy' and 'kettle hop' flavour upon addition to a non-aromatised iso-α-acid bittered lager beer (see **Chapter 2** and **4**). It is hypothesised that during boiling of hops in real brewing practice, such oxidation products might also be formed. 'Kettle hop' flavour might (at least partly) originate from elevated terpene oxidation product levels in beer.

In this chapter, we aim at verifying our results obtained on a lab scale in real brewing practice. To this end, four different conventionally aromatised lager beers were prepared at our pilot-scale brewery and exclusively hopped with a noble hop variety (cv. Saaz), varying the point of hop addition ('early', 'late', 'whirlpool' hopping, and a combination of 'early' and 'late' hopping, respectively). Samples were taken along the wort boiling and whirlpool process, and analysed via HS-SPME-GC-MS, aiming at obtaining insights into the behaviour of hop oil-derived volatiles during these processes. In addition, we prepared a non-aromatised iso-α-acid bittered brew, which was split post-fermentation. From the five resulting brews, one remained non-aromatised, whereas the other four were 'dry' hopped. One beer was dry-hopped with pellets, whereas three beers were aromatised by addition of respectively unboiled total hop essential oil, boiled total hop essential oil and a SOP fraction cv. Saaz, prepared as described in **Chapter 4**. To investigate the impact of the hopping regime on the 'hoppy' flavour in all beers, sensory evaluation by our trained taste panel was performed.

It is a well-known fact that analysis of hop-derived volatiles in beer is a challenging task, taking into account the low levels at which these constituents are detected and co-elution with major fermentation products. Therefore, we also applied multidimensional gas chromatography mass spectrometry (GCxGC-TOFMS) in order to investigate which hop-derived volatiles are present in the beer aromatised with the SOP fraction (cv. Saaz).

6.2 Experimental

6.2.1 Chemicals

The following reference compounds were purchased from Sigma-Aldrich (St. Louis, MO) and were of analytical grade: 2-decanone (99.5%); 2-dodecanone (97.0%); 2-heptanol (98%); 2-nonanone (99.5%); 2-tridecanone (97.0%); 2-undecanone (99.0%); caryophyllene oxide (≥99.0%); decanal (≥98.0%); geraniol (≥99.0%); limonene (97.0%); linalool (98.5%); methyl 3-nonenoate (99.8%); methyl decanoate (99.5%); methyl geranate; methyl nonanoate (99.8%); methyl octanoate (99.8%); nerol (≥97.0%); ocimene (≥90.0%, mixture of isomers); p-cymene (≥99.0%); terpinen-4-ol (≥95.0%); terpinolene (≥90.0%); *trans*-β-farnesene (≥90%); α-copaene (≥90%); α-humulene (≥98.0%); α-pinene (98.0%); β-caryophyllene (≥98.5%); β-damascenone (≥98.0%); β-ionone (≥97.0%); β-myrcene (≥95.0%); β-pinene (99.0%); γ-terpinene (≥97.0%). Iso-caryophyllene and oxygenated sesquiterpenoid mixtures of reference compounds were prepared as described in **section 2.2.1.2**.

Ethanol absolute (EtOH) (≥99.8%) was purchased from VWR International (Zaventem, Belgium); MQ water was obtained using a MQ purification system (Synergy 185, Millipore S.A., Molsheim, France); Sodium chloride was purchased from Merck (for analysis, 1 kg, Darmstadt, Germany).

6.2.2 Plant material

Saaz hop pellets T90 (crop year 2014) were provided by the Barth-Haas Group (Joh. Barth & Sohn GmbH & Co. KG, Nürnberg, Germany). For storage conditions, see **section 2.2.2**.

6.2.3 Hop oil content determination via steam distillation

The hop oil content of T90 pellets cv. Saaz was determined according the EBC method 7.10 (EBC Analytica, hops and hop products, hop oil content of hops and hop products) using steam distillation. There proved to be 0.50 mL hop oil per 100 g pellets (n=8, CV= 0.3%). Isolation of hop essential oil for further use in brewing was performed as described in **section 2.2.3.1.**

6.2.4 Preparation of boiled and unboiled total hop essential oil and a SOP fraction (cv. Saaz) for advanced aromatisation of pilot-scale lager beers

6.2.4.1 Boiled and unboiled total hop essential oil

For preparation of boiled total hop essential oil, high hop oil concentrations were chosen to promote *de novo* formation of SOPs (see **Chapter 5**). To HS-SPME vials containing 4,940 µL MQ-water was added 58.8 µL hop oil cv. Saaz, aiming at a concentration of 10 g/L. Vials were boiled for 1 h in the incubation oven of the CombiPAL autosampler (CTC Analytics, Zwingen,

Switzerland) (see **section 5.2.4**). After cooling of the samples, the EtOH concentration was increased (15 mL EtOH was added to each vial) to 75% to promote solubility and dispersion of hop oil volatiles in wort, resulting in a hop oil concentration of 2.5 g/L. Analogously, an unboiled hop essential oil solution (2.5 g/L unboiled hop oil in 75/25 EtOH/MQ-water; v/v) was prepared.

6.2.4.2 Sesquiterpene oxidation product (SOP) fraction

A sesquiterpene hydrocarbon (SHC) fraction cv. Saaz was obtained via steam distillation of pellets cv. Saaz, followed by SPE, as described in **section 4.2.4**. HS-SPME vials containing 1 g/L SHC fraction in MQ-water were boiled in the incubation oven of the CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland) and oxidation products formed *de novo* upon boiling were enriched via SPE as described in **section 4.2.6.** The fraction eluting with 70% EtOH is indicated as the SOP fraction. Using a caryophyllene oxide external calibration curve (10 points, ranging form 0 to 2.5 mg/L) (slope: 68.211, intercept: 0.0375, R^2: 0.9921), the oxygenated sesquiterpenoid level in the SOP fraction was determined at 167 mg/L.

6.2.5 Preparation of pilot-scale lager beers

Five brews were prepared at the pilot brewery (5-hL scale) of KU Leuven (lab EFBT, Technology Campus Ghent, Belgium). The brewing installation is a prototype for innovative wort production as described by De Rouck *et al.*[224]. For brewing, the following conditions were used: 87 kg fine milled Pilsner malt (wet disc mill, Meura, Péruwelz, Belgium) is mixed with 2.5 hL reversed osmosis brewing water with addition of $CaCl_2$ (80 ppm Ca^{2+}) and lactic acid (2 mL/L); mashing-in: temperature: 64°C; pH 5.2; brewing scheme: 64°C (30 min), 72°C (20 min), 78°C (1 min) (temperature increase: 1°C/min); wort filtration: membrane assisted thin bed filter; sparging up to 11.5°P sweet wort; wort boiling: 60 min atmospheric boiling using a double jacket for heating (evaporation: 5%); addition of iso-α-acid extract (except for beer E) aiming at a total level of 25 ppm iso-α-acids in the finished beers and 0.2 ppm Zn^{2+} ions end boiling; wort clarification: whirlpool; after cooling and aeration, the wort (original gravity: 12°P) was pitched with 10^7 yeast cells/mL (inoculum: dry yeast, strain KO5 (Fermentis), hydrated for 1 h in sterile water with a volume of 10 times the weight of the dry yeast); primary fermentation: 9-13 days at 12°C in cilindroconical tanks; maturation: 14 days at 0°C in 50 L casks; beer filtration: kieselguhr/cellulose sheets (pore size 1 µm); CO_2 saturation up to 5.6 g/L; packaging: 6 head rotating counter pressure filler (monobloc, CIMEC, Italy) using double pre-evacuation with intermediate CO_2 rinsing and overfoaming with hot water injection before capping (final oxygen levels: below 50 ppb).

Four brews were conventionally hopped by addition of hop pellets (noble hop variety cv. Saaz) to the boiling kettle, whereas one brew was exclusively bittered with pre-isomerised

iso-α-acid extract as described below. In order to understand the impact of the hopping procedure on the hop oil-derived spectrum of volatiles and flavour characteristics of the resulting beer, the point of hop addition of the 4 conventionally hopped lagers was varied (hop additions standardised by weight), whereas all other parameters were kept constant. Beer E was hopped with 300 g/hL Saaz pellets at the onset of boiling ('early kettle hopping'), aiming at a final iso-α-acid concentration in the beer of 25 mg/L (taking into account an initial α-acid content of 2.37% (w/w) in the hops (on the basis of HPLC analysis) and a utilisation of 35%). For the late hopped beer (beer L), an equal amount of hop pellets (300 g/hL) was added 10 minutes before the end of wort boiling and iso-α-acid extract (20% iso-α-acids, presumed utilisation of 65%)(Botanix, Paddock Wood, England) was added to compensate for the bitterness (7.1 mg pellet-derived iso-α-acids/L based on a utilisation of 10%; addition of 13.762 g isomerised extract/hL resulting in 17.9 mg iso-α-acids/L). A combination of these two hopping regimes was obtained by addition of 150 g pellets/hL at the onset and 150 g pellets/hL towards the end of boiling (beer EL: 'early' and 'late' hopping) (16.0 mg iso-α-acids/L, derived from pellets). For compensation of the bitterness, 6.925 g isomerised extract/hL was added, resulting in 9.0 mg iso-α-acids/L. Finally, a beer (beer W) was bittered exclusively by addition of 16.496 g isomerised hop extract/hL to the kettle (resulting in 21.4 mg iso-α-acids/L) and then aromatised by 'whirlpool' hop addition (300 g/hL pellets; 3.6 mg iso-α-acids/L based on utilisation of 5%).

The last brew, which was not aromatised but bittered with iso-α-acid extract (19.231 g iso-extract/hL, resulting in 25 mg iso-α-acids/L), was split post-fermentation into 5 different casks for lagering (50 L each). One beer was not aromatised and served as the reference beer (beer 'ISO'). The second beer (50 L) was 'dry hopped' by addition of pellets (100 g/hL) in the cask, resulting in beer D-pellets. For aromatisation of the third, fourth and fifth beer (50 L), advanced hopping via addition of resp. unboiled hop oil dilution (20 mL), boiled hop oil dilution (20 mL), and SOP fraction (112 mL) (see **section 6.2.4**) in the cask was carried out, resulting in three beers containing resp. 1 mg unboiled hop oil/L (beer D-U), 1mg boiled hop oil/L (beer D-B) and 312.5 µg SOP fraction/L (beer D-SOP) at the onset of lagering. Upon addition of pellets and hop oil (fractions), the casks were shaken for homogenisation of the content.

6.2.6 Sampling along the brewing process

Samples (500 mL) were taken along the boiling process of beer E and during the whirlpool stage of beer W for analysis of hop-derived volatiles. For all conventionally hopped beers (beer E, EL, L and W), samples were taken at the end of wort boiling and at the end of the whirlpool process. Chemical reactions were immediately stopped by cooling the samples in

liquid nitrogen (–196°C), and samples were kept frozen (–18°C) until further HS-SPME-GC-MS analysis. For a detailed overview of all samples taken for the different beers, see **Table 6-1**.

Table 6-1. Overview of samples taken along the brewing process of different beers.

 = hop addition (T90 pellets cv. Saaz).  = addition of hop essential oil-derived fraction. X= sampling

Samples	Beer E	Beer EL	Beer L	Beer W	Beer D-pellets	Beer D-U	Beer D-B	Beer D-SOP
0 min, before hopping	x							
Early hop addition								
5 min of boiling	x							
10 min of boiling	x							
20 min of boiling	x							
30 min of boiling	x							
40 min of boiling	x							
50 min of boiling	x							
Late hop addition								
60 min of boiling (end boiling)	x	x	x	x				
Transfer to whirlpool								
Whirlpool hop addition								
0 min whirlpool (start whirlpool)				x				
5 min whirlpool				x				
10 min whirlpool				x				
15 min whirlpool				x				
20 min whirlpool (end whirlpool)	x	x	x	x				
Fermentation								
End fermentation								
Dry-hopping								
Start lagering								
Lagering								
End lagering								
Filtration								
Post-filtration								
Final beer	x	x	x	x	x	x	x	x

6.2.7 HS-SPME-GC-MS analysis

Levels of oxygenated sesquiterpenoids (OSs) in worts and beers were estimated by external calibration using the reference compound caryophyllene oxide. The 8-point calibration curve ranged from 0 to 50 µg/L (1 g NaCl, 5% EtOH, 20 µL internal standard stock solution (2-heptanol, 253 mg/L), 0 to 50 µL caryophyllene oxide stock solution (5,000 µg/L)). Using this calibration curve, levels of OSs can be expressed in caryophyllene oxide equivalents.

Wort and beer samples were analysed by adding 5 mL sample and 20 µL internal standard (2-heptanol, 253 mg/L stock solution) in a HS-SPME vial (20 mL, clear glass, Chromacol)

containing 1 g NaCl. Vials were closed with bimetal magnetic caps with silicon/Teflon septum (Supelco, Bellefonte, USA).

Hop-derived volatiles were extracted via headspace solid-phase microextraction (HS-SPME) (fibre coating: polydimethylsiloxane (PDMS), extraction time: 45 min, extraction temperature: 60°C, splitless injection) as previously described in **section 2.2.6**. Gas chromatographic conditions for separation of the volatiles were described in **section 2.2.6**. For chemical-analytical characterisation of wort and beer samples, slow oven programming was used. For determination of the level of OSs in the pilot-scale lager beers, fast oven programming was used. Mass spectrometric detection of volatiles was performed as described in **section 2.2.6**.

6.2.8 Comprehensive GC – Time of Flight Mass Spectrometry (GCxGC-TOFMS)

The SOP-fraction (see section **6.2.4**) and beer D-SOP and D-ISO, were analysed via GCxGC-TOFMS analysis. To a HS-SPME vial containing 4,970 µL MQ-water was added 25 µL SOP fraction and 5 µL internal standard (2-heptanol, 12.5 g/L stock solution). Beers were analysed by addition of 5 mL beer and 20 µL internal standard (2-heptanol, 253 mg/L stock solution) to a HS-SPME vial containing 1 g NaCl. For headspace solid phase microextraction of the volatiles, the vials were placed in a water bath (60°C) and a PDMS fibre (100 µm) was inserted through the septum and exposed to the heaspace. After 45 minutes, the fibre was withdrawn and manually injected in the GCxGC-TOFMS system (splitless injection).

Analysis was performed using a py-GCxGC-TOFMS system. The GCxGC-TOFMS used is a Pegasus 4D (Leco, USA), which consists of an Agilent Technologies 6890N gas chromatograph equipped with a secondary oven, a nonmoving 2 stage thermal modulator, and a Pegasus III time-of-flight mass spectrometer. Modulation was performed with N_2 (Air liquide, Belgium), which was supplied via an automatic filler (Leco corporation, Lakeview avenue, St.-Joseph, USA) with liquid level controller (AMI model 186).

The used GC columns are a RTX-1 100% dimethyl polysiloxane (30 m x 0.32 mm i.d., 0.25 µm film thickness) as first dimension column, coupled to a BPX-50 50% phenyl polysilphenylene-siloxane (1.3 m x 0.10 mm i.d., 0.10 µm film thickness) as second dimension column. The second dimension column is directly connected to the TOF-MS. The target flow was set at 0.8 mL/min during the entire run (constant flow mode). The oven program of the first column was as follows: 3 min at 35°C, ramp of 6°C/min up to 250°C, hold for 5 min. For the second column, the oven program was as follows: 3 min at 45°C, ramp of 6°C/min up to 260°C, hold for 5 min. Modulation was performed every 6 seconds (hot pulse time: 0.8 s, cool time between stages: 2.20 s). After separation, the analytes are transferred to the mass spectrometer using a transferline at 320°C.

The MS detector operated in Electron Ionisation mode (IE) using an Ion source temperature of 250°C, electron energy of -70 Volts, a detector voltage of 1700 Volts and a scan range from 29-500 m/z with an acquisition rate of 100 Hz.

Data acquisition and processing were performed using ChromTOF® software version 3.25 (Leco, USA). After acquisition, the sample data are processed using automatic peak detection using a signal-to-noise ratio of 500:1, and automated peak integration based on the deconvoluted total ion current signal. Identification of peaks is performed by comparing the deconvoluted mass spectra to the NIST mass spectra library software (NIST MS Search version 2.0g and database version 2011), to reference mass spectra found on the NIST website (http://webbook.nist.gov/chemistry/), and to mass spectral information from books[188,189].

6.2.9 Sensory evaluation of lager beers by taste panel

In first instance, the significance of sensory differences among the reference beer (beer ISO) and aromatised lager beers (beer E, EL, L, W, D-pellets, D-U, D-B and D-SOP), and among beer E and beers EL, L and W, were investigated by the trained taste panel of our institute (8 panellists) via triangular tests (α-level: 0.05). During each (separate) triangular test (11 tests in total), 3 samples were served (randomised order) and panellists were asked to indicate the different sample.

Subsequently, in separate sessions, odour and aroma characteristics of the lager beers were evaluated via descriptive sensory analysis by our trained taste panel. The panel was trained using reference compounds, total hop essential oils and hop-derived essences (total hop oils, polar, floral, citrus and spicy essences prepared as described by Van Opstaele *et al.*[175,184]), and commercially available hop oil fractions (PHA® Spicy, Citrusy, Floral, Herbal and Sylvan (Botanix, U.K.)). Each aromatised lager was compared to the non-aromatised reference lager (beer ISO). Panel members were instructed to score the intensity of pre-selected descriptors (malt/worty, fruity, floral, citrusy, spicy/herbal, woody, hay/straw, resinous, grass/green, earthy, intensity of 'kettle hop aroma', general appreciation, bitterness, quality bitterness, mouthfeel and astringency) on a scale ranging from 0 to 8 (0=not detectable, 8=very high intensity).

6.3 Results and discussion

6.3.1 Investigation of the full hop oil-derived volatile spectrum along the brewing process: from boiling to whirlpool

6.3.1.1 Evolution of hop oil-derived volatiles during wort boiling

In order to gain insight into the impact of the 'kettle' hopping regime on the analytical composition of the hop-oil derived spectrum of volatiles in the wort, the evolution of hop-derived compounds throughout the brewing process of an 'early kettle' hopped beer (beer E) was investigated. Samples were taken at different points during the wort boiling process (see **Table 6-1**) and the volatile composition was determined using HS-SPME-GC-MS analysis. Peak areas of chemical compound classes (monoterpene hydrocarbons, floral fraction (*i.e.* ketones, esters, alcohols, oxygenated monoterpenoids)[123], SHCs, and spicy fraction (*i.e.* ketones, esters, alcohols, OSs)[124]) were normalised and average normalised peak areas (duplicate analysis) are plotted in **Figure 6-1 A**.

Obviously, 'early' kettle hopping gives rise to both mono- and sesquiterpene hydrocarbons, as well as floral and spicy compounds in the wort. Levels of monoterpene and sesquiterpene hydrocarbons clearly decrease with increasing boiling time, due to known processes such as stripping and probably polymerisation. Compounds within the floral fraction also show a decrease. Although these compounds are better soluble in wort compared to terpene hydrocarbons, these molecules are still relatively volatile which could explain their losses. *De novo* formation of oxygenated monoterpenoids by oxidation of monoterpene hydrocarbons can not be excluded, since losses due to volatilisation could (over)compensate for increases, resulting in a net decrease. Remarkably, spicy compounds show a rather low but significant increase in their level with increasing boiling time, starting from 20 minutes of boiling. This is an interesting observation that might be explained by long extraction times (*i.e.* slow transfer of these volatiles from hop pellets into the wort), and/or by oxidation of SHCs into OSs. *De novo* formation of OSs during wort boiling has amply been suggested in literature[10,16,30,39,83,113,134,157]. Also in the previous chapters, *de novo* formation has been proven to occur during lab scale boiling experiments. However, up to date, it has not been demonstrated during real brewing practice. In an attempt to confirm the observed data, wort was brewed in an identical way (same malt, brewing parameters and hopping regime as beer E; hopped wort was, however, not fermented in this case). **Figure 6-1 B** confirms the results discussed above, *i.e.* an increase in the level of spicy compounds (incl. OSs) with increasing wort boiling time in real brewing practice.

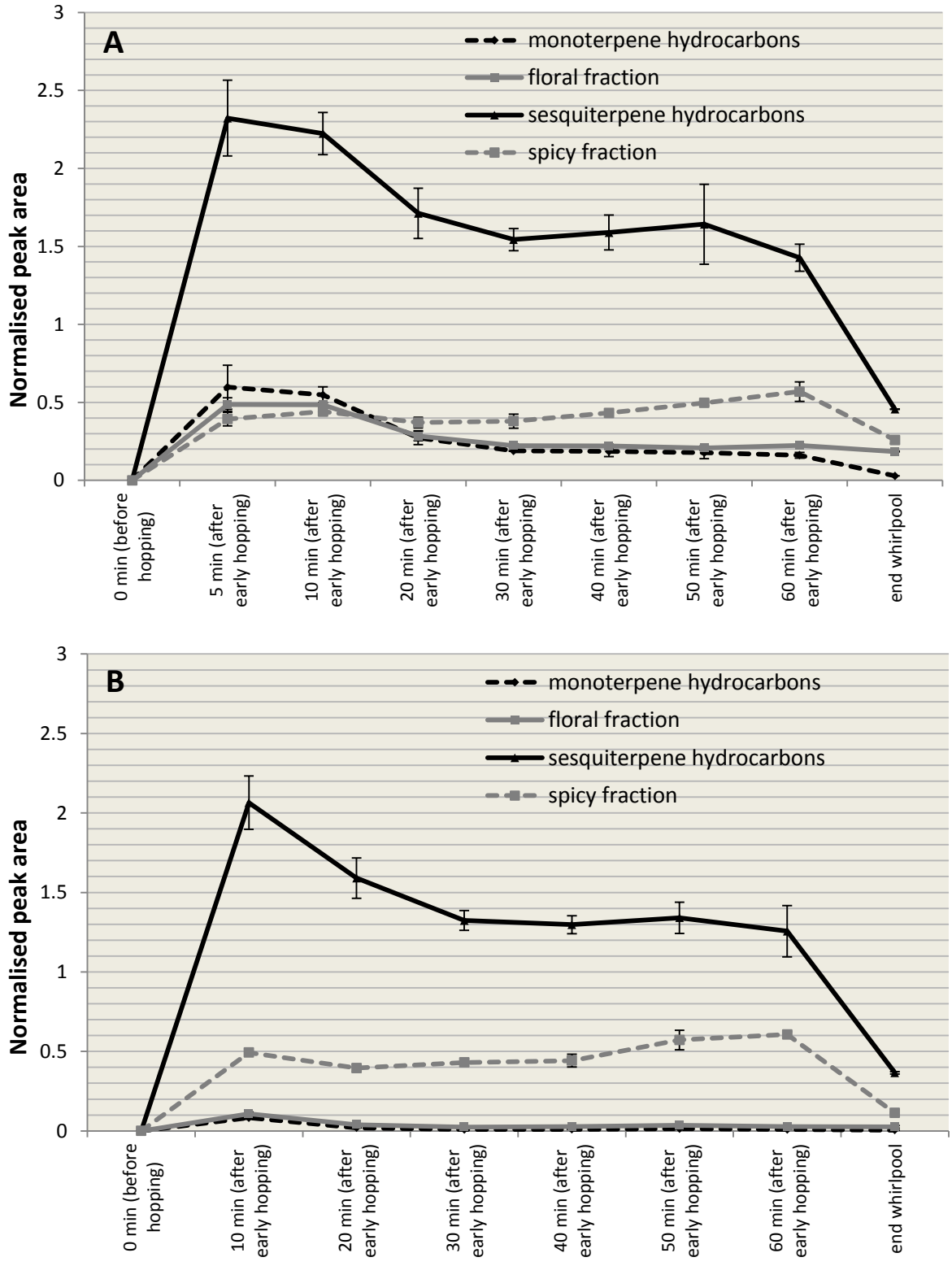

Figure 6-1. Average normalised peak area for different chemical compound classes of hop oil (-derived) volatiles, detected via HS-SPME-GC-MS analysis, as a function of samples taken along the wort boiling process and at the end of the whirlpool stage of brew E ('early' kettle hopping with cv. Saaz). A= results of brew E. B= results of replicate of brew (parameters as for brew E, this wort was however not fermented).

To verify as to which extent this increase may concern *de novo* formation of OSs, we looked for differences in the behaviour of SOPs (*e.g.* epoxides and their hydrolysis products) and OSs that are related to the hop plant metabolism (*e.g.* cadinols[18]). As found in previous chapters (see **Chapter 2**,**4** and **5**), the latter group did not increase in level during lab scale boiling of total hop essential oil (cv. Saaz) or a hop oil-derived SHC fraction (cv. Saaz, Super Pride). On the other hand, a significant increase in levels of α-humulene and β-caryophyllene oxidation products was demonstrated.

τ-Cadinol, α-cadinol and several α-humulene and β-caryophyllene oxidation products were selected amongst the spicy compounds as marker compounds. For each volatile, the normalised peak areas in the different samples was expressed as a percentage of the normalised peak area found after 5 minutes of boiling. The resulting recoveries (%) are displayed in **Figure 6-2** and depict the evolution of the selected marker compounds with increasing boiling time. In **graph A**, the evolution of the cadinols is shown. Levels reach a maximum after 10 minutes, which might be the extraction time required for these compounds. A further increase with increasing boiling time is however not observed. On the contrary, the α-humulene and β-caryophyllene oxidation products (also showing a first maximum after 10 min of boiling) show a significant increase in their levels with increasing boiling time (see **graph B**). Caryophyllene oxide and humulene epoxide II show less pronounced increases in their level. This particular finding confirms our previous lab scale results (see **Chapter 2**) and can be explained by the fact that these epoxides are relatively prone to hydrolysis and rearrangement reactions[10,17,18,85,138].

Since there is clear indication for *de novo* formation of several compounds during wort boiling, a comprehensive profiling of hop-derived volatiles was performed (see **Table 6-2)**. The recovery of each detected volatile was estimated via comparison of the normalised peak area obtained after 50 min of boiling to areas obtained after 5 min of boiling. Because of the risk of co-elution of volatiles in the HS-SPME-GC-MS-derived chromatograms, peak areas were determined in the SIM (selected ion monitoring) mode. This allows for selection of specific and unique mass fragments and thus more accurate determination of changes in levels during wort boiling.

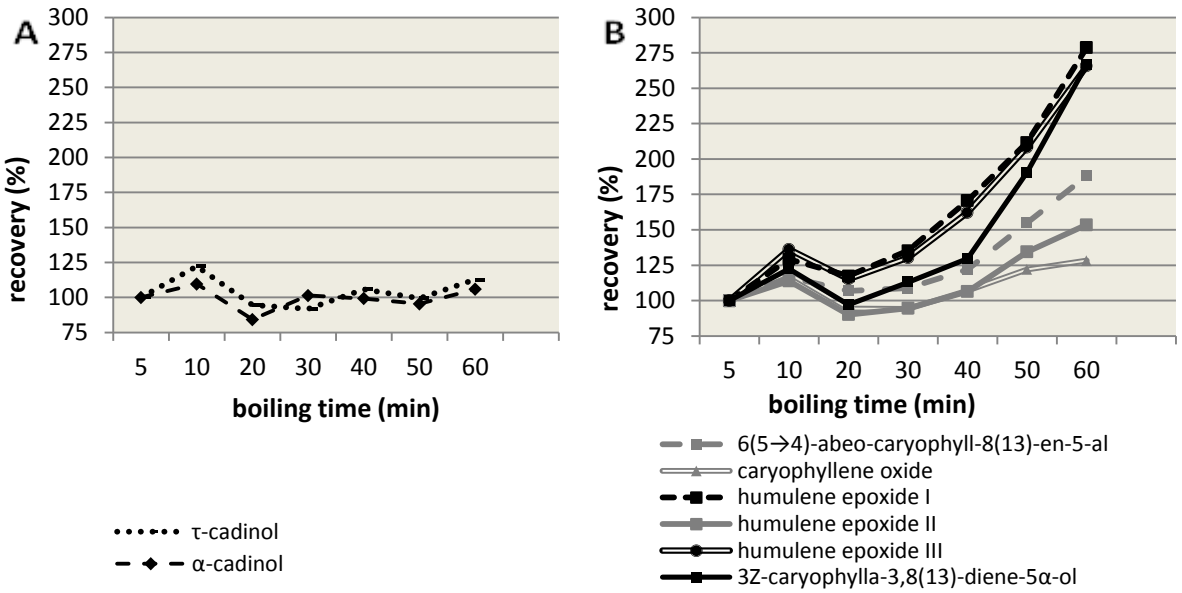

Figure 6-2. Recovery (on basis of average normalised areas, determined in SIM mode) of selected cadinols (A) and α-humulene and β-caryophyllene oxidation and hydrolysis products(B) upon wort boiling of brew E (recovery in %, relative to normalised peak area found after 5 minutes of wort boiling).

Table 6-2. Tentative identification and recoveries (%) of volatiles detected in samples from wort boiling of brew E. RI= retention index (calculated on RTX-1 column). SIM= selected ion monitoring (selection of specific characteristic mass fragments for accurate determination of normalised peak areas), full scan= m/z 40-250. R(%)= recovery, based on normalised SIM peak areas (sample after 50 min of boiling vs. 5 min of boiling). Identification based on MS (mass spectrum), RI (retention index), RC (reference compound) or comparison with mixtures of reference compounds (IEP/CEP/HEP= *iso*-caryophyllene/caryophyllene/humulene epoxide, CAA/HAA= caryophyllene/humulene allylic alcohol, HHP/CHP= humulene/caryophyllene hydrolysis product, see section 2.2.1.2). N= detected after 50 min of boiling but not detected after 5 min of boiling. Bold= increase in level of the volatile upon wort boiling.

Compound	RI	SIM mass fragments	R (%)	Identification
β-Pinene	<1000	69, 93	26	MS/RI/RC
β-Myrcene	<1000	69, 93	29	MS/RI/RC
p-Cymene	**1002**	**119, 134**	**156**	**MS/RI/RC**
Unknown (m/z 55, 82, 110, 111, 127, 142)	1011	55, 82, 110, 111, 127, 142	0	
Limonene	1022	68, 93	27	MS/RI/RC
Trans-β-ocimene	1040	91, 93	34	MS/RI/RC
Methyl 6-methylheptanoate	1073	74, 87	0	MS/RI
2-Nonanone	1079	58	0	MS/RI/RC
γ-Terpinene	1081	93, 121, 136	82	MS/RI/RC
Hop ether	1085	122, 137	35	MS
Linalool	1086	71, 93, 121	41	MS/RI/RC
Perillene	1089	69, 81, 150	53	MS/RI
Karahana ether	1091	79, 122	61	MS
2-Decanone	1175	58, 71	15	MS/RI/RC
α-Terpineol	1176	81, 93, 121, 136	99	MS/RI
Ethyl octanoate	1183	88, 101, 127	98	MS/RI
Dodecene	1189	55, 69, 83, 97, 111	65	MS/RI
Methyl 3-nonenoate	1197	74, 96, 138	92	MS/RI/RC
Methyl nonanoate	1211	74, 87	27	MS/RI/RC
Geraniol	1239	69	60	MS/RI/RC
Methyl ketone	1239	58, 71	36	MS
Ethyl ester	1247	88, 101	48	MS
Unknown (m/z 69)	1257	69	45	
5-Undecen-2-one	1257	43	25	MS/RI
2-Undecanone	1274	58, 71	42	MS/RI/RC
Methyl 4-decenoate	1291	74, 110, 152	35	MS/RI
Unknown (m/z 85)	1294	85	33	
Methyl geranate	1303	69, 114, 123	33	MS/RI/RC
Methyl ester	1311	74, 87	52	MS

Table 6.2 continued

β-Damascenone	**1361**	**69, 121, 190**	**272**	MS/RI/RC
α-Ylangene	1367	Full scan	87	MS/RI
α-Copaene	1371	Full scan	93	MS/RI/RC
2-Dodecanone	1376	Full scan	68	MS/RI/RC
Ethyl decanoate	1381	88, 101	81	MS/RI
Tetradecene	1389	Full scan	74	MS/RI
Unknown (m/z 79, 81, 80, 122)	1394	Full scan	48	
Isocaryophyllene	1400	Full scan	90	MS/RI/RC
***Cis*-α-bergamotene**	**1408**	**93, 119**	**104**	MS/RI
β-Caryophyllene	1412	Full scan	84	MS/RI/RC
β-Copaene	1421	Full scan	93	MS/RI
Trans-α-bergamotene	1429	93, 119	92	MS/RI
Unknown oxygenated sesquiterpenoid (m/z 69, 81, 95, 109, 123, 138, 149, 191, 205, 220)	**1438**	Full scan	**145**	MS
α-Humulene/β-farnesene	1446	Full scan	72	MS/RI/RC
β-Ionone	**1462**	**177**	**137**	MS/RI/RC
Trans-cadina-1,4-diene	1463	204	66	MS/RI
γ-Muurolene	1465	204	86	MS/RI
α-Amorphene	1469	204	82	MS/RI
Unknown oxygenated sesquiterpenoid (m/z 69, 81, 95, 109, 123, 138, 149, 191, 205, 220)	**1473**	**191, 205 220**	**137**	MS
β-Selinene	1475	204	95	MS/RI
Neryl isobutanoate	1475	69	99	MS/RI
2-Tridecanone	1476	58, 71	78	MS/RI/RC
Cis-cadina-1,4-diene	1482	204	87	MS/RI
α-Selinene	1485	204	93	MS/RI
Epi-zonarene	1486	204	64	MS/RI
α-Muurolene	1489	204	89	MS/RI
(E,E)-α-Farnesene	1496	204	85	MS/RI
β-Bisabolene	1499	204	80	MS/RI
γ-Cadinene	1501	204	82	MS/RI
Trans-calamenene	1505	159	93	MS/RI
δ-Cadinene	1510	204	73	MS/RI
Zonarene	1512	204	57	MS/RI
Trans-cadina-1,4-diene	1519	204	70	MS/RI
α-Calacorene	1523	142, 157, 200	90	MS/RI
α-Cadinene	1524	204	91	MS/RI
4S-Dihydrocaryophyllene-5-one	**1530**	**79, 96, 109, 138, 164, 220**	**211**	MS/RI
Isocaryophyllene epoxide A	**1531**	**106**	**N**	MS/RI/IEP
4R-Dihydrocaryophyllene-5-one	**1534**	**79, 96, 109, 138, 164, 220**	**275**	MS/RI
β-Calacorene	1541	142, 157, 200	81	MS/RI
Unknown (m/z 79, 80, 81, 150, 157)	1541	79, 80, 81	88	
Unknown oxygenated sesquiterpenoid (m/z 93, 107, 121, 205, 220)	**1544**	**93, 205, 220**	**219**	MS
Humuladienone	**1550**	**67, 96, 109, 138**	**135**	MS/RI
Caryolan-1-ol	**1550**	**111**	**130**	MS/RI
6(5→4)-Abeo-caryophyll-8(13)-en-5-al	**1556**	**79, 93, 107, 121, 164, 205, 220**	**162**	MS/RI
E-Dendrolasin	**1556**	**69, 81**	**215**	MS/RI
Caryophyllene oxide	**1560**	Full scan	**119**	MS/RI/CEP
Clovenol	**1563**	**161, 205, 220**	**117**	MS/RI/CHP
Gleenol	1567	81, 121	89	MS/RI
Unknown (m/z 107, 135, 218)	1567	107, 135, 218	99	MS
Humulene epoxide I	**1574**	**93**	**206**	MS/RI/HEP
Humulol	**1579**	**82, 83**	**163**	MS/RI/HHP
Humulene epoxide II	**1585**	**96, 109, 138**	**133**	MS/RI/HEP
Humulene allylic alcohol	**1593**	**105, 107, 109, 159, 177, 205, 220**	**158**	MS/RI/HAA
1,10-Di-epi-cubenol	1595	119, 161, 179, 204	80	MS/RI
Humulene epoxide III	**1606**	**81**	**307**	MS/RI/HEP
Humulenol II	**1608**	**119**	**115**	MS/RI/HAA
Caryophylla-4(12),8(13)-diene-5-ol	**1613**	**136**	**154**	MS/RI/CAA
τ-Cadinol	1618	161	42	MS/RI
Cubenol	1623	161	84	MS/RI
α-Cadinol	1631	95, 121	97	MS/RI
3Z-Caryophylla-3,8(13)-diene-5α-ol	**1634**	Full scan	**187**	MS/RI/CAA
Unknown (m/z 79, 80, 81)	1636	Full scan	99	
Unknown	1639	Full scan	90	
3Z-Caryophylla-3,8(13)-diene-5β-ol	**1649**	Full scan	**152**	MS/RI/CAA
Cadalene	1648	183, 198	88	MS/RI
Humulene allylic alcohol	**1655**	Full scan	**136**	MS/RI/HAA

From **Table 6-2**, it can be seen that most hop-derived volatiles show a recovery lower than 100%. This observation does not exclude *de novo* formation during boiling, since potential increases in levels might not be detected due to losses by phenomena, such as adsorption to trub and stripping effects. However, a number of volatiles prove to increase in level upon boiling. p-Cymene, a disproportionation product of limonene[136], is detected amongst these volatiles. In addition, the β-carotene oxidative degradation products β-damascenone and β-ionone are also found to increase in level upon wort boiling. An increase in the β-damascenone level during wort boiling was previously observed by Kishimoto and coworkers[20]. With respect to sensory properties, the odour of β-damascenone (flavour threshold: 0.009 µg/L[88]) has been described as 'apple, peach' and 'honey-like'[11,166]. The same volatile was also perceived during GC-O sniffing analyses of Pilsner beer by Fritsch and Schieberle[166] and GC-O analysis of both unhopped beer and beers hopped with cv. Challenger and cv. Saaz by Lermusieau and coworkers[11]. The dilution factor at which β-damascenone could be detected was however clearly higher in the hopped beers. On the other hand, it has been suggested that β-ionone does probably not influence beer hoppy character since it was not perceived upon GC-O analysis of beer[11]. Nevertheless, β-ionone (flavour threshold: 0.008 µg/L[88]) has been described as 'floral' and 'violet-like'[12,77], and, both β-damascenone and β-ionone are present in beer at levels at which they may contribute to the aroma[147]. In this respect, the observed increases in their levels during wort boiling might be relevant to beer aroma.

Strikingly, all of the detected α-humulene and β-caryophyllene oxidation products show a recovery higher than 100%, suggesting *de novo* formation of these compounds during wort boiling by oxidation of their parent SHC molecule. On the contrary, cadinols and cubenols do not show a recovery higher than 100%. Amongst the SHC oxidation products, isocaryophyllene epoxide was not detected in the samples taken after 5 min of boiling, whereas it was found in samples taken after 50 minutes of boiling. This indicates that also qualitative changes in the hop oil-derived volatile profile may occur, as a result of boiling hops.

Typical 'noble kettle hop aroma', achieved by 'early' addition of aroma hop varieties, which are usually rich in α-humulene, is described by 'spicy' and 'herbal' notes. A cause-and-effect relationship between SOPs and these odour characteristics has been shown by us via addition of the SOP fraction to a non-aromatised iso-α-acid bittered lager beer (see **Chapter 4**). Moreover, many of the SHC-derived oxidation products were found to elute in flavour-active intervals, detected upon GC-O analysis of spicy fractions obtained by SPE-fractionation of a commercial kettle hopped lager beer (see **Chapter 3**). Increases in α-humulene and β-caryphyllene oxidation products, previously found to occur during lab scale boiling, have

now also been demonstrated during the wort boiling process in real brewing practice by monitoring hop oil-derived volatiles of an 'early' kettle hopped lager beer. Therefore, it can be concluded that boiling of aroma hops definitely alters the hop oil composition and that *de novo* formation of SOPs may play a key role in the development of 'kettle hop' aroma of beer.

6.3.1.2 Evolution of hop oil-derived volatiles during wort clarification

The impact of 'whirlpool hopping' was investigated by HS-SPME-GC-MS analysis of wort samples taken along the whirlpool process of beer W (see **Appendix D**). Normalised peak areas of chemical compound classes are depicted in **Figure 6-3**, showing that terpene hydrocarbons as well as oxygenated compounds are extracted into the wort via whirlpool hopping. However, terpene hydrocarbons are lost to a great extent, which could be attributed to volatilisation and adsorption to hot break. Losses of oxygenated compounds appear to be less pronounced, due to their higher solubility in wort. Nevertheless, a general increase in the level of spicy compounds, as was detected during wort boiling, was not found during the whirlpool stage.

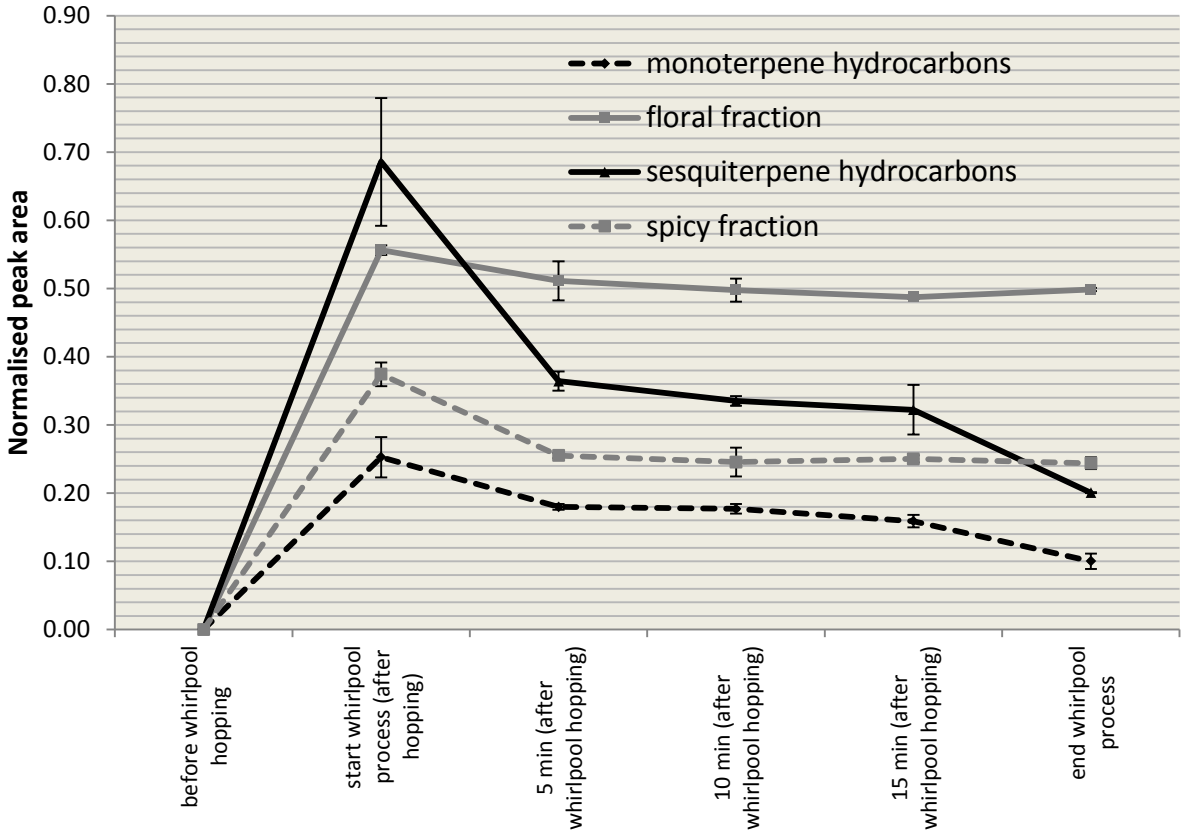

Figure 6-3. Average normalised peak area for different chemical compound classes of hop oil (-derived) volatiles, detected via HS-SPME-GC-MS analysis, as a function of samples taken along the whirlpool process of brew W ('whirlpool' hopping with cv. Saaz).

The full spectrum of volatiles was determined via HS-SPME-GC-MS analysis of samples taken at the start and the end of the whirlpool stage of brew W (see **Appendix D**). Although some of the detected volatiles are (at least partly) wort-derived (*i.e.* they also appear in samples taken before hopping, such as phenylacetaldehyde, borneol, vinylguaiacol, β-damascenone), the major part is derived from the hop essential oil. Clearly, whirlpool hopping gives rise to a broader spectrum of volatiles in the wort compared to 'early kettle hopping' since many monoterpenoid compounds, not detected in the wort samples of beer E, are now detected in the wort samples of beer W. Some examples of such compounds are dihydro-ocimene, myrcenol, terpinen-4-ol, nerol and several unidentified monoterpenoids. The absence of these compounds in the wort samples of beer E can be rationalised by stripping effects since temperatures in the boiling kettle are higher than in the whirlpool. A series of compounds was characterised by an increase in their level during the whirlpool stage, although the recoveries of most of these compounds are only slightly higher than 100%.

To investigate whether these increases are due to slight increases in levels as a function of the whirlpool time or are rather due to variation, the evolution of these volatiles along the whirlpool stage was monitored into more detail by determination of the normalised peak areas in each sample (start, 5 min, 10 min, 15 min and end whirlpool) in the SIM-mode and plotting as a function of whirlpool time.

The unknown monoterpenoid at RI 1060, borneol (RI 1146), an unknown at RI 1156, an unknown at RI 1183, and geraniol (RI 1235) showed recoveries between 106 and 123% (see **Appendix D**). From **Figure 6-4 A**, it remains doubtful whether the level of these volatiles actually increases during the whirlpool stage or not.

The behaviour of α-terpineol (RI 1171), nerol (RI 1211), 4 unknowns (at RI 1142, 1257, 1264 and 1381), and humulol (RI 1574) is depicted in **Figure 6-4 B**, indicating that these volatiles increase in their level during the whirlpool stage.

Finally, the evolution of volatiles showing the highest increase in their level during whirlpooling is depicted in **Figure 6-4 C**. β-damascenone, which increases in level during wort boiling, appears to further increase during the whirlpool stage. Also 4-vinylguaiacol shows some increase, although this volatile is wort-derived[225]. The norisoprenoid dihydroedulan shows a clear increase as reflected by the recovery of 265% after 20 minutes in the whirlpool. This rather atypical compound was identified for the first time in a glycosidic extract form Saaz spent hops and hopped beer by Daenen[22]. The increase in the level of dihydroedulan (as well as terpineol, geraniol and nerol) may originate from glycosidically bound volatiles in hops. Also β-damascenone can be derived from glycoconjugated precursors after acid catalysed conversion[22]. Finally, also the behaviour of

myrcenol, a β-myrcene-derived monoterpene alcohol, previously detected in the oil of hops by Gildemeister and Hoffman[226], suggests *de novo* formation during the whirlpool step.

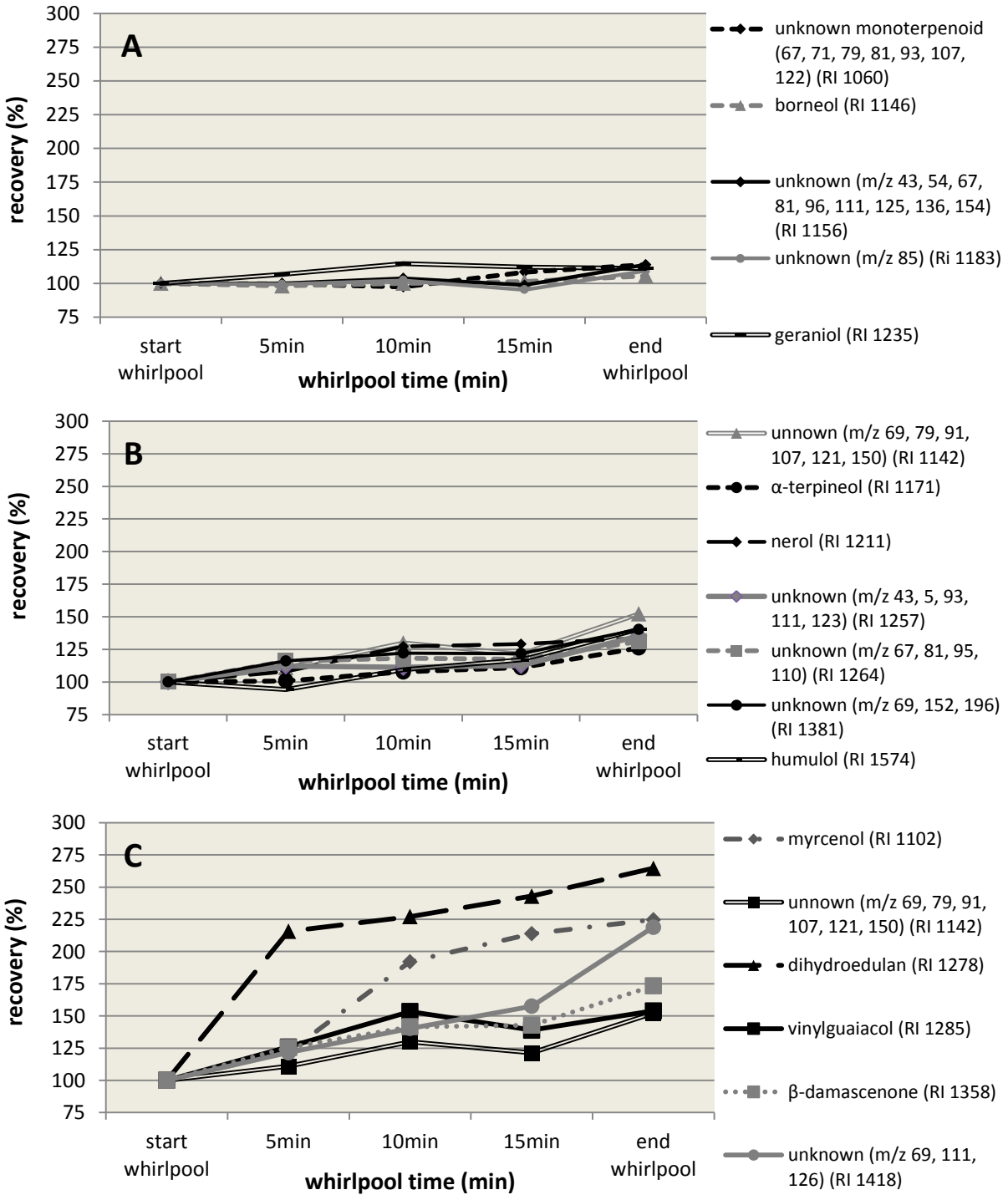

Figure 6-4. Recovery (based on average normalised areas, determined in the SIM mode) of volatiles during the whirlpool stage of brew W (recovery in %, relative to normalised peak area found at the start of the whirlpool stage). Volatiles in graph B show a slight increase in their level, whereas volatiles in graph C show a clear increase in their level, probably due to *de novo* formation.

Although the identity of many volatiles remains unknown, it is clear that monoterpenoid alcohols (such as myrcenol, borneol, α-terpineol, nerol, geraniol) and norisoprenoids (β-

damascenone, dihydroedulan) are amongst the volatiles that increase in level during wort clarification. These compounds are most probably formed by thermal oxidation of monoterpene hydrocarbons and degradation of carotenoids, due to the relatively high remaining temperature of the wort (90 °C) at the whirlpool stage. Also release of glycosidically bound volatiles might explain observed increases of particular volatiles during the whirlpool process. An increase in the level of SOPs, which was clearly detected during wort boiling, is not found during the whirlpool stage. Temperatures in the whirlpool are possibly not high enough for significant oxidation of SHCs or, in case some oxidation would occur, formation of these volatiles could be quickly compensated by losses due to adsorption to trub.

In summary, it can be concluded that the whirlpool process also induces changes in the volatile hop oil-derived fingerprint. Yet, the analytical profiles of 'early kettle' hopped wort and 'whirlpool hopped' wort are clearly different from both a quantitative and qualitative point of view. As a result, it can be expected that beer E and beer W will show clearly different flavour characteristics.

6.3.2 Determination of OS levels in pilot-scale hopped lager beers

Since OSs are believed to be important with respect to 'kettle hop' aroma, OSs levels in the different beers were determined semi-quantitatively. To this end, levels of OSs in beers were expressed as caryophyllene oxide equivalents using a caryophyllene oxide calibration curve (see **section 6.2.7**) (regression coefficient R^2: 0.9962). The OS levels are depicted in **Figure 6-5** and, for the aromatised beers, levels range from 6 ppb (beer D-pellets) to 39 ppb (beer D-SOP).

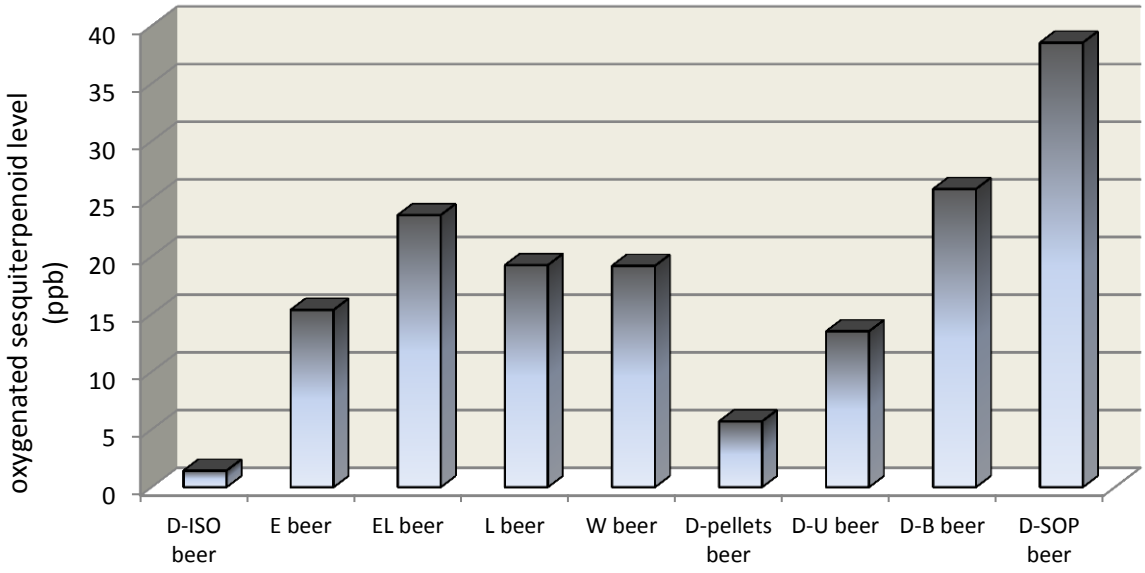

Figure 6-5. Levels of oxygenated sesquiterpenoids (OSs) (ppb) in reference (D-ISO), conventionally (E, EL, L and W) and advanced aromatised (D-pellets, D-U and D-B) pilot-scale lager beers

Taking into account the relatively high hopping rates for beer EL, L and W (300 g pellets /hL wort for each beer; range according to the EBC manual Hops and Hop Products: 50-600 g/hL for early hopping and 20-40 g/hL for 'mid', 'late' or 'whirlpool' additions[1]), OS levels may at first sight seem relatively low. However, at most 12.8 ppm of hop oil was introduced into the wort for each beer, due to low hop oil contents in Saaz hops (0.5 mL/100g). Moreover, a large proportion of total hop essential oil consists of SHCs and ketones (up to 90%[43,51,160]) that may not survive the brewing process (caused by processes such as volatilisation, polymerisation, adsorption to trub/yeast and migration to the foam layer[10,16,90,113,133,141,148]). The hopping rate for the beer dry-hopped with pellets (beer D-pellets) is within the normal range (100 g/hL; range according to the EBC manual Hops and Hop Products: 17-170 g/hL for dry hopping[1]) and would introduce at most 4.25 mg/L hop oil into the beer. On the other hand, addition dosages of unboiled hop oil, boiled hop oil and the SOP fraction are at the lower end (resp. 1 ppm, 1 ppm and 312.5 ppb; hop oil addition according to EBC manual Hops and Hop Products: *e.g.* 1 to 5 ppm[1]).

Levels of oxygenated terpene compounds in lager beer have been reported at 15-50 ppb[133,157]. Also in **Chapter 4**, levels of OSs in commercial lagers (exhibiting distinct kettle hop flavour) were estimated at 33 to 109 ppb. Taking into account these values, the OS levels in our beers lie within the normal range. However, despite the relatively low level at which OSs are found, these compounds may have a significant impact on the hop-derived flavour of beer. Indeed, OSs have been reported to be detectable up to levels as low as 5.8 ppb upon addition to beer[157]. Also Goiris and coworkers determined the flavour threshold of an enriched OS hop essence in beer at 5 ppb[19]. However, levels of 20 ppb where preferred and introduced a pleasant, spicy hop flavour and enhanced mouthfeel, fullness and bitterness perception, whereas higher addition rates were described as overwhelming for pilsner beer types[19]. Later on, addition of a hop-derived spicy essence to beer confirmed these findings[184]. In this respect, it appears that our applied hopping rates resulted in an OS level which might find itself within the ideal concentration range to impart subtle, yet balanced 'kettle hop' flavour.

Clearly, from all aromatised beers, beer D-pellets contains the lowest OS level (**Figure 6-5**), confirming that dry hopping is not an efficient technique when 'spicy/herbal' notes are desired in the final beer. Post-fermentation addition of unboiled hop oil seems far more effective towards transfer of OSs to the beer. Remarkably, addition of an identical concentration of boiled hop oil significantly increases OS levels and could therefore be a new and promising technique with regard to post-fermentation introduction of 'spicy/herbal' flavour. Beer D-SOP clearly contained the highest OS level. Nevertheless, large amounts

were lost during lagering and filtration since, from the 312.5 ppb OSs introduced at the start of lagering, only 39 ppb was recovered in the final beer.

Of the 4 conventionally aromatised beers, beer E proved to contain the lowest OS level. However, in **section 6.3.1**, it was found that 'early' addition of hop pellets leads to an increase in the spicy fraction during boiling, due to *de novo* formation of SOPs and, subsequently, one would expect this beer to have increased OS levels compared to the other beers. Therefore, normalised peak areas of spicy compounds in hopped samples at the end of the boiling process (start whirlpool process for beer W), at the end of the whirlpool stage, and in the final beer, were plotted in **Figure 6-6**. From this graph, it can be concluded that levels of spicy compounds in beer E are indeed elevated at the end of the boiling process and, furthermore, the later hop pellets were added, the lower are the levels of spicy compounds at this stage. However, spicy compounds are lost to a great extent during the whirlpool stage and also during subsequent process steps, such as fermentation, lagering and beer filtration. Higher percentages of losses of spicy compounds in brew E (54% loss during whirlpool stage of brew E versus 43%, 28% and 2% losses for resp. brews EL, L and W) and subsequent stages, due to adsorption to hop vegetative matter, hot and cold break, and yeast cells, may explain why beer E is characterised by the lowest OS level.

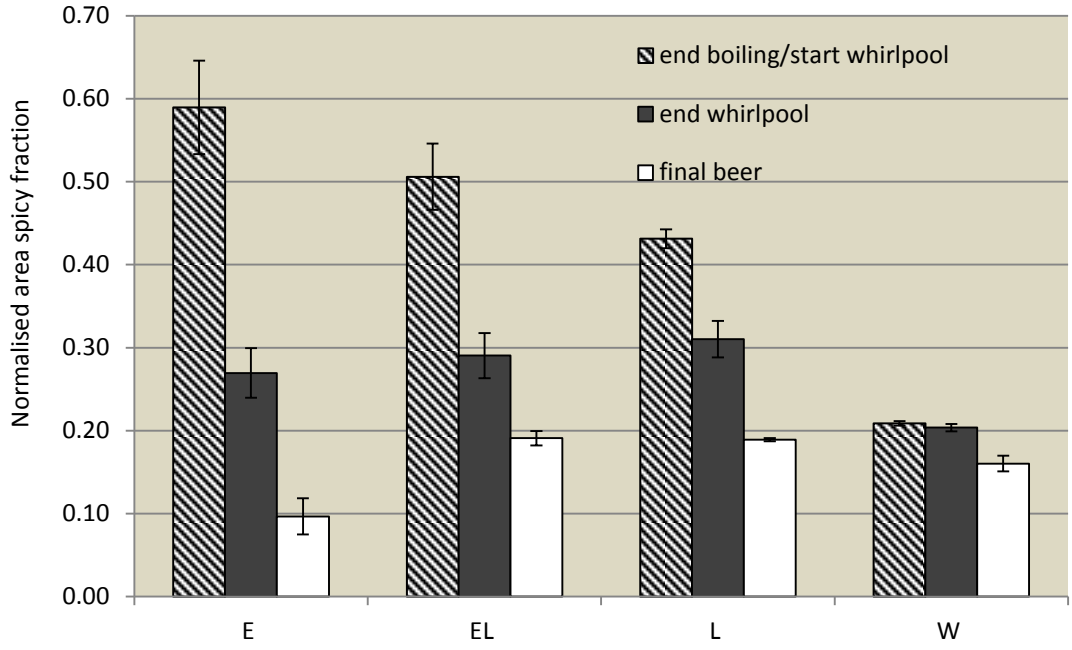

Figure 6-6. Normalised peak area (n=2) of spicy fraction in samples taken at the end of the boiling process, at the end of the whirlpool stage and in the final beer for beers E, EL, L and W.

6.3.3 Sensory evaluation of hopped lager beers

6.3.3.1 Triangular tests

Via a first series of triangular tests, sensory differences between the non-aromatised iso-α-acid-bittered beer (D-ISO) and the aromatised beers were investigated. The results showed that all of the applied hopping technologies imparted significant sensory differences (α-level: 5%) compared to the non-aromatised reference beer. Also, although some brewers believe 'early kettle' hopping does not cause hop-derived aroma because all hop volatiles would be stripped out of the wort, addition of aroma hops at the onset of boiling clearly introduces hop-derived aroma in beer E.

Furthermore, via a second series of triangular tests, sensory differences between beer E and the beers EL, L and W were determined (α-level: 5%). This finding confirms that addition of hops at the onset of wort boiling or later on in the process (even during the whirlpool stage) clearly results in beers with different flavour attributes and that the point of hop addition definitely has an impact on 'hoppy' aroma. Although levels of hop oil-derived volatiles remaining in the final beers are relatively low (ppb range, see also **section 6.3.2**), these quantities are obviously sufficient to impart distinct hop-derived flavour characteristics to lager beer.

6.3.3.2 Descriptive tests

Evaluation of general appreciation, kettle hop and dry hop characteristics

Via descriptive sensory evaluations, panellists were asked to score general appreciation for the different beers from 0 to 8 (score 0= not appreciated; score 8= highly appreciated), and to give an intensity score for the preselected odour/aroma descriptors 'kettle hop aroma' and 'dry hop aroma'. In **Figure 6-7**, it is shown that hopping with Saaz pellets or hop oil (-derived fractions), regardless of the applied hopping regime, consistently resulted in higher appreciation scores when compared to the unhopped ISO beer. Beer EL, hopped by addition of Saaz pellets both at the onset and towards the end of wort boiling, received the highest appreciation. Such a hopping regime is frequently applied in brewing practice and is characteristic for a classic Pilsner type beer.

Addition of unboiled hop oil during lagering resulted in the least appreciated beer amongst the aromatised beers. However, boiling of hop oil prior to addition to the cask slightly increases general appreciation. The beer aromatised by post-fermentation addition of SHC-derived oxidation products (D-SOP) received the second highest general appreciation score. Although this new type of hop oil-derived fraction was created off-line, aromatising with this

particular fraction appears to impart positive flavour attributes to lager beer and may therefore show potential for application in real brewing practice.

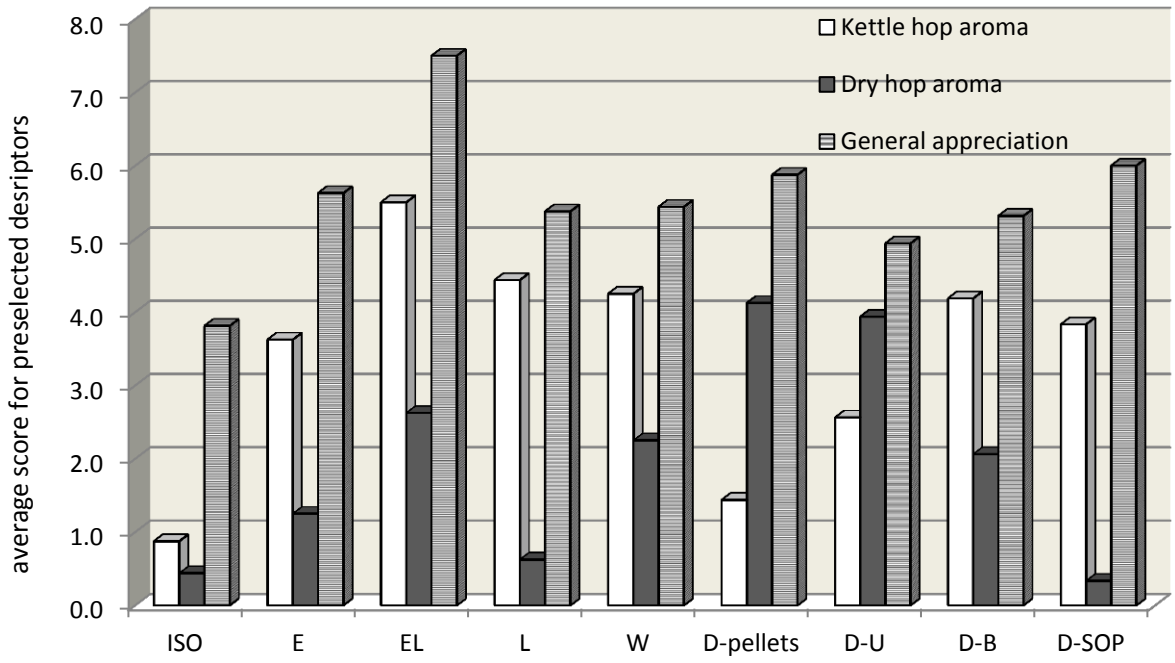

Figure 6-7. Average score (n=8) for general appreciation, kettle hop aroma and dry hop aroma for the reference beer and 8 aromatised lager beers. ISO= non-aromatised iso-α-acid bittered lager beer, E= early kettle hopping, EL= early and late hopping, L= late hopping, W= whirlpool hopping, D-pellets= dry hopping, D-U= addition unboiled hop oil, D-B= addition boiled hop oil, D-SOP= addition sesquiterpene oxidation product fraction.

Evidently, conventionally hopped beers received a relatively high score for 'kettle hop' aroma, whereas the score assigned to 'dry hop' aroma is remarkably lower. For the dry-hopped beers D-pellets and D-U the reverse is observed. However, the beers dry-hopped with boiled hop oil or the SOP fraction (resp. D-B and D-SOP) were scored significantly higher for 'kettle hop' aroma, although kettle hopping was obviously not part of the brewing procedure. These fractions are thus promising towards application in brewing practice in order to impart 'kettle hop' aroma flavour characteristics at the post-fermentation stage.

Evaluation of odour/aroma characteristics via various descriptors

The average of the scores assigned for the various descriptors for each hopped beer is compared to the scores given for the reference ISO beer in separate spider plots (see **Figure 6-8** and **Figure 6-9**).

Figure 6-8 A depicts the flavour profile of beer E, showing that 'early kettle' hopping impacts the flavour of lager beer by masking 'malty' and 'worty' flavours. Also 'fruity' flavours, which are in general caused by fermentation esters, slightly decreased as a consequence of 'early kettle' hopping. 'Floral', 'citrusy', 'grass/green' and 'resinous' notes are detected and especially flavour attributes described as 'spicy/herbal' and 'woody' are expressed as a

consequence of 'early kettle hopping'. The remarkable increase in 'spicy/herbal' and 'woody' aromas compared to the ISO beer can be explained by the presence of OSs, which confirms our previous study, in which unhopped iso-α-acid-bittered lager beer demonstrated 'spicy/herbal' and 'woody' aroma upon addition of a SOP fraction (see **Chapter 4**).

Beer EL (see **Figure 6-8 B**), for which a portion of the hop pellets was added 'early' in the boil and another portion 'late' in the boil, also shows these 'spicy/herbal' and 'woody' flavours. In addition, as can be expected from the late hop addition, scores for the descriptors 'floral', 'citrusy' and 'grass/green' are significantly higher compared to the ISO beer, and also beer E. Beer EL expresses both the 'spicy/herbal' aromas typical for 'noble kettle hop' aroma, and 'floral/citrus' notes, which might explain why this beer was so highly appreciated by the panellists.

The flavour profile of beer L (**Figure 6-8 C**) shows that addition of all hop pellets towards the end of wort boiling did not lead to elevated scores for 'floral' and 'citrus' compared to beer EL. Moreover, 'grass/green' and 'fruity' notes were scored much lower and also the 'spicy/herbal' aroma was less pronounced. On the other hand, panellists detected an increased 'woody' aroma in beer L.

The flavour profile of beer W (**Figure 6-8 D**), exclusively hopped during the whirlpool stage, is somewhat comparable to that of beer EL, although 'spicy/herbal' notes are less pronounced and a strong 'resinous' aroma is noticed. Compared to beer L, beer W shows stronger 'grass/green' and 'resinous' aromas. Panellists specifically mentioned that beer W was comparable to beer EL, but, also agreed that the distinct 'resinous' aroma had a rather negative impact on general appreciation for beer W.

Although beer E contains relatively low OS levels compared to the other conventionally aromatised beers (EL, L and W) (see **Figure 6-5**), beer E was scored relatively high for 'spicy/herbal' notes. This observation might be explained by less masking due to less 'late hop' flavours (floral, citrusy) in beer E. Linalool, for example, has been proven to be a contributor to the floral aroma of beer[11,12,14,166]. On the basis of the normalised peak area of linalool, it could be concluded that the beers EL, L and W contain resp. 2.7, 3.7 and 4 times more linalool than beer E and also the descriptor 'floral' was scored significantly higher in these beers. Accordingly, the expression of 'spicy/herbal' notes, characteristic for 'noble kettle hop' aroma in lager beer, might not solely be dependent on the absolute level of flavour-active OSs present, but rather on the ratio of volatiles imparting 'floral' aroma and 'spicy' aroma, respectively. The ratio of spicy compounds versus linalool was calculated on the basis of standardised peak areas, resulting in a ratio of 29, 25, 17 and 14 for beer E, EL, L

and W, respectively. Clearly, the beers E and EL show a higher ratio and also the descriptor 'spicy/herbal' was scored significantly higher for these beers.

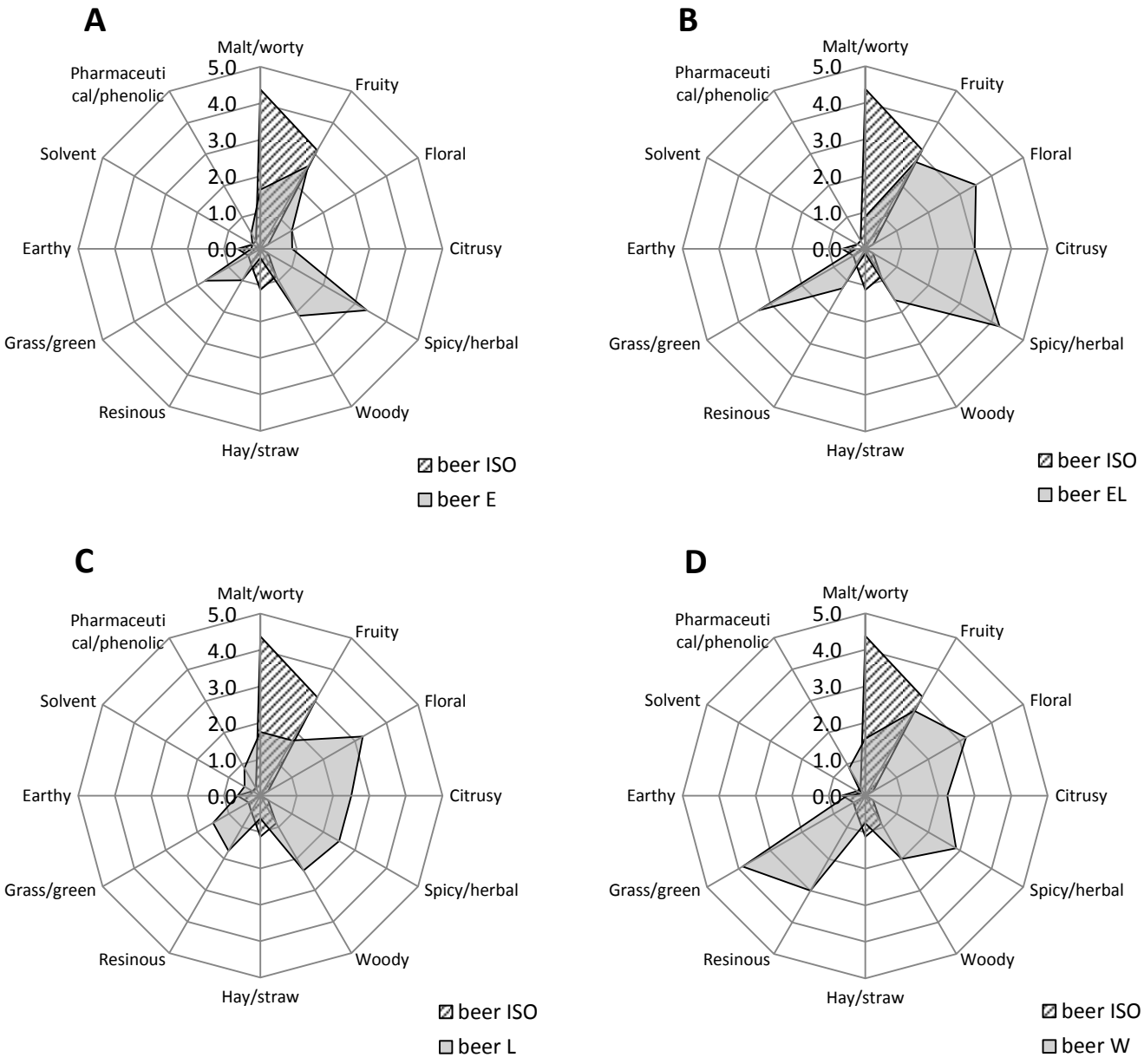

Figure 6-8. Spider plots depicting the flavour profile of beer E, beer EL, beer L and beer W (resp. graph A, B, C and D) compared to a non-aromatised beer, bittered with iso-α-acids (ISO), based on the average score (8 panellists) for pre-selected odour/flavour descriptors.

In beer D-pellets, grassy notes are clearly detected (**Figure 6-9 A**). Malty and worty flavours were suppressed, whereas 'floral', 'citrusy' and 'spicy' attributes clearly came to expression. Since the OS level in this beer is relatively low (see **Figure 6-5**), the spicy note might be related to the presence of hop ketones[165]. Panellists also mentioned that this beer received a relatively high score for appreciation due to its pleasant 'citrusy' and 'floral' bouquet.

The flavour profile of beer D-U (**Figure 6-9 B**), dry hopped by addition of hop essential oil, is similar to that of beer D-Pellets. However, strong resinous notes, which were not perceived

in beer D-pellets, are detected in beer D-U. Beer D-U received the lowest score for appreciation among all aromatised beers. Panellists specified that, although the dry-hop odour of the beer was pleasant, the strong resinous character and also other flavour attributes (bitterness, astringency, mouthfeel) had a negative impact, resulting in a beer that is out of balance.

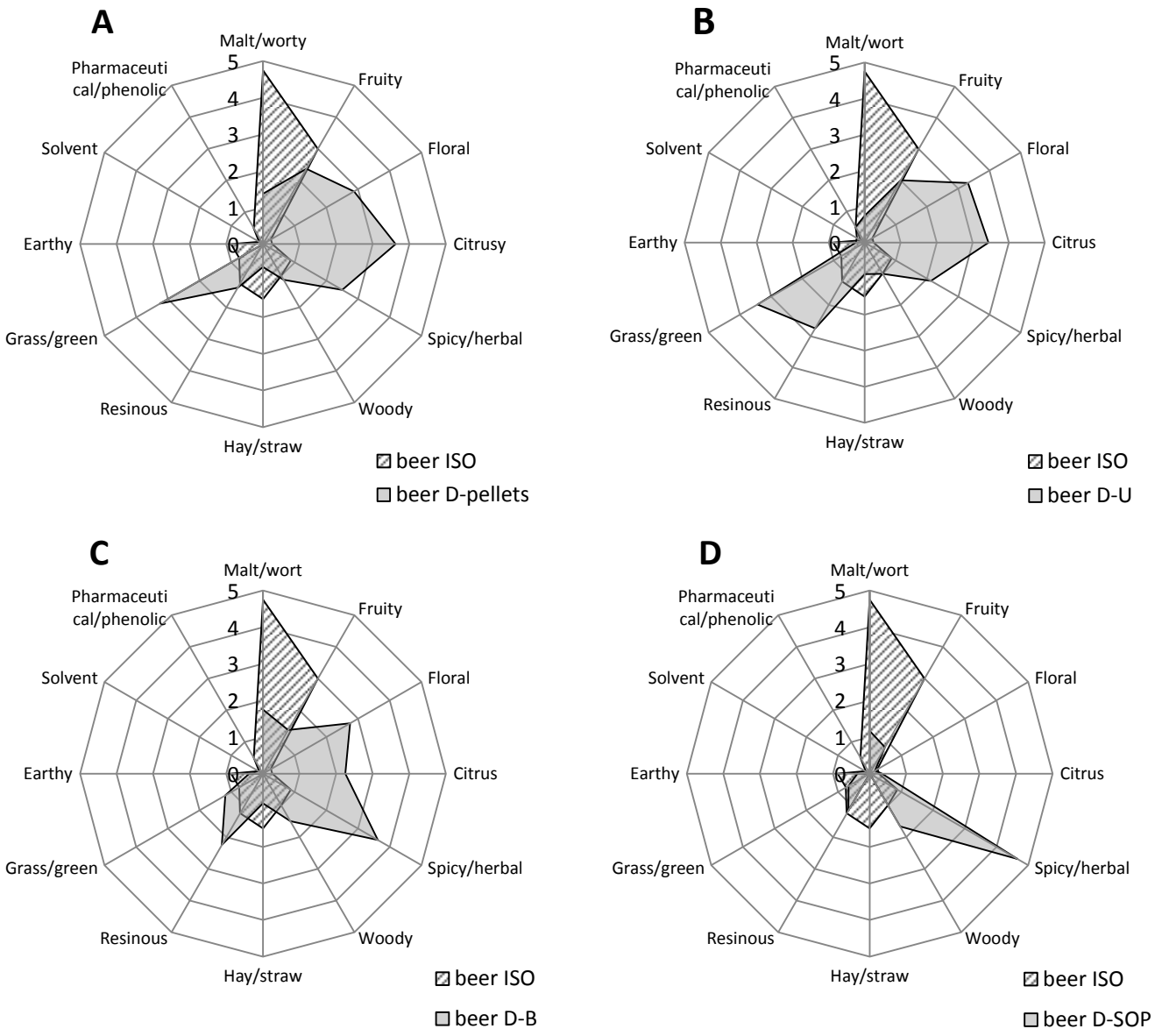

Figure 6-9. Spider plots depicting flavour profile of beer D-pellets, beer D-U, beer D-B and beer D-SOP (resp. graph A, B, C and D) compared to a non-aromatised beer, bittered with iso-α-acids (ISO), based on average score (8 panellists) for pre-selected odour/flavour descriptors.

Boiling of hop essential oil prior to addition to the cask clearly alters the flavour profile of the resulting beer (beer D-B, see **Figure 6-9 C**). Grassy and green notes are hardly perceived, and also 'floral' and 'citrusy' notes are found less intense. On the other hand, kettle hop aroma is pronounced in beer D-B, which is reflected by a signigicantly higher score for 'spicy/herbal'

flavour. These results confirm our findings in **Chapter 2**, where it was noticed that non-aromatised iso-α-acid bittered beer spiked with boiled hop oil exhibited 'spicy' flavour and 'kettle hop' aroma. Nevertheless, panellists found the beer D-B sliglthly out of balance and proposed that this could be due to the relatively high dosage of boiled hop oil. Therefore, beer D-B was diluted 1/1 (v/v) with the non-aromatised reference beer. All panellists agreed that upon dilution, a well balanced beer with 'kettle hop' flavour was obtained. It was concluded that beer D-B shows the desired 'kettle hop' aroma characteristic for a classic Pilsner-type beer.

The flavour profile of beer D-SOP (**Figure 6-9 D**) is quite similar to the flavour profile of beer E (**Figure 6-8 A**), since the aroma is dominated by 'spicy/herbal' notes and, to a lesser extent, 'woody' notes. The comparable flavour profiles of beer D-SOP and E can be explained by the fact that SOPs constitute the largest part of hop-derived volatiles in both beers. The absence of oxygenated monoterpene alcohols may further clarify the lack of 'floral' and 'citrusy' notes in both beers. Panellists underlined that beer D-SOP definitely expresses an aspect of 'kettle hop' aroma. However, 'kettle hop' flavour was found incomplete and less complex compared to beer D-B.

Evaluation of taste and mouthfeel

Scores for other flavour attributes, such as bitterness intensity and quality, mouthfeel and astringency for all beers are summarised in the spider plots shown in **Figure 6-10**. Except for the quality of bitterness in the beers D-U, D-B and D-pellets, all flavour attributes were scored higher in the aromatised beers. Most remarkably is the increase in mouthfeel and bitterness quality upon addition of the SOP-fraction, which confirms results from previous studies of EFBT during wich hop oil sesquiterpenoid fractions appeared to positively influence mouthfeel aspects (in particular fullness) and bitterness perception[15,19,184]. The same effect is, albeit to a slightly lesser extent, also imparted by post-fermentation addition of boiled hop essential oil.

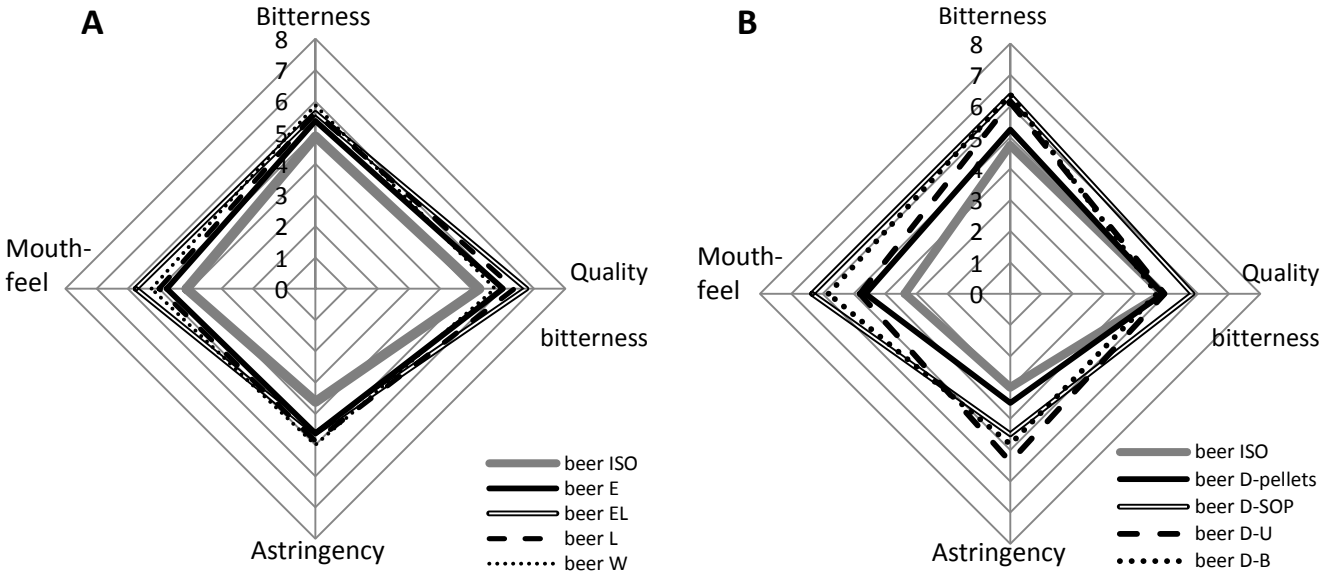

Figure 6-10. Spider plots depicting bitterness quality and intensity, mouthfeel and astringency of beer E, EL, L, W (graph A), and of the beers D-pellets, D-U, D-B and D-SOP (graph B) compared to a non-aromatised beer, bittered with iso-α-acids (ISO), based on average score (8 panellists) for the pre-selected flavour descriptors.

Conclusions

In summary, beer EL was described as having the most intense 'kettle hop aroma' and was also the most appreciated beer. Although beer E clearly showed the 'spicy/herbal' notes typical for 'noble kettle hop' aroma, panellists agreed that the hop-derived aroma of beer EL was more complex, which can be explained by the additional late hopping. Panellists also concluded unanimously that among the dry-hopped beers, beer D-B was most reminiscent to a classic kettle-hopped Pisner-type beer, which points to potential of boiled hop oil for application in brewing practice. Although beer D-SOP also expressed the typical 'spicy/herbal' flavour note, this beer was described as less complex and 'kettle hop' flavour was found incomplete. Apparently, 'spicy/herbal' notes alone, caused by sesquiterpene oxidation prodcuts, are not sufficient and other flavour-active hop oil-derived volatiles are also required to obtain the full 'ketlle hop' aroma characteristic. It seems that relatively high OS levels, combined with a subtle amount of 'floral/citrus' odourants, explains the high general appreciation attributed to the beers EL and D-B and their 'noble kettle hop' aroma characteristics. Addition of a portion of rather expensive aroma hops at the onset of boiling seems to make sense since the 'spicy/herbal' notes in both beers E and EL were highly appreciated by the panellists, although these flavour attribrutes can be mimicked by post-fermentation addition of SOPs or boiled hop essential oil.

6.3.4 GCxGC-TOFMS analysis of the SOP fraction and of a beer aromatised by post-fermentation addition of the SOP fraction

In **Chapter 4**, it was found that SHC-derived oxidation products impart 'spicy/herbal' notes when spiked to a non-aromatised iso-α-acid bittered lager beer and this result was confirmed in real brewing practice. Moreover, the beer prepared in **Chapter 4** clearly expressed 'kettle hop' flavour. GC-O analyses on the SOP-fraction revealed intense flavour-active zones, comprising caryophylla-3,8(13)-diene-5α-ol, 14-hydroxy-β-caryophyllene and caryophylla-3,8(13)-diene-5β-ol. In literature, the latter compound has been reported as flavour-impact compound in an ale beer[146], whereas 14-hydroxy-β-caryophyllene was previously detected in hop oil[114]. As reported in **Chapter 3**, these constituents were also detected in our study when analysing commercial kettle hopped beers via SPE enrichment of the spicy fraction. Detection and identification of these volatiles in beer remains however challenging, due to their extremely low levels, highly similar mass spectral fingerprint (see **Chapter 4**) and co-elution with other volatiles when performing monodimensional GC. To overcome the problem of co-elution and to unequivocally demonstrate the presence of potentially important flavour-impact sesquiterpenoids in both the SOP-fraction and beer D-SOP, 2D gas chromatography – mass spectrometry (GCxGC-TOFMS) was performed.

GCxGC-TOFMS analysis of the SOP-fraction

The SOP-fraction was subjected to profound GCxGC-TOFMS analysis for comprehensive fingerprinting of the volatile profile. A 3D and 2D plot of the SOP-fraction are depicted in **Figure 6-11 A** and **B**, respectively. **Figure 6-11 C** depicts a part of the 2D plot into more detail, showing the most intense peaks in the spicy region of the SOP-fraction. Each circle in the plot points to peak detection by the software (peak colour and circle radius indicate the peak intensity (level of the respective compound) and are scaled to the highest peak in the selected part of the plot). Clearly, many detected peaks are present in trace amounts.

Table 6-3 gives an overview of compounds with relative area > 0.05% and the corresponding (tentative) identity of the compounds, determined on the basis of comparison of deconvoluted mass spectra to reference mass spectra. We were able to identify a high number of compounds, among which several terpenes, floral compounds, and spicy compounds that are not related to oxidation of SHCs. For example, the major terpene hydrocarbons β-myrcene, β-caryohyllene, α-humulene and β-farnesene make up 26.5% of the total SOP-fraction. These compounds and other constituents present at trace level are 'impurities' of the SOP-fraction and their presence can not be prevented, due to limitations of SPE fractionation. However, the SOPs isocaryophyllene epoxide A, caryophyllene oxide, humulene epoxide I, humulene epoxide II and humulene epoxide III account for 45.7% of the fraction, pointing to strong enrichment of SOPs in the SOP-fraction.

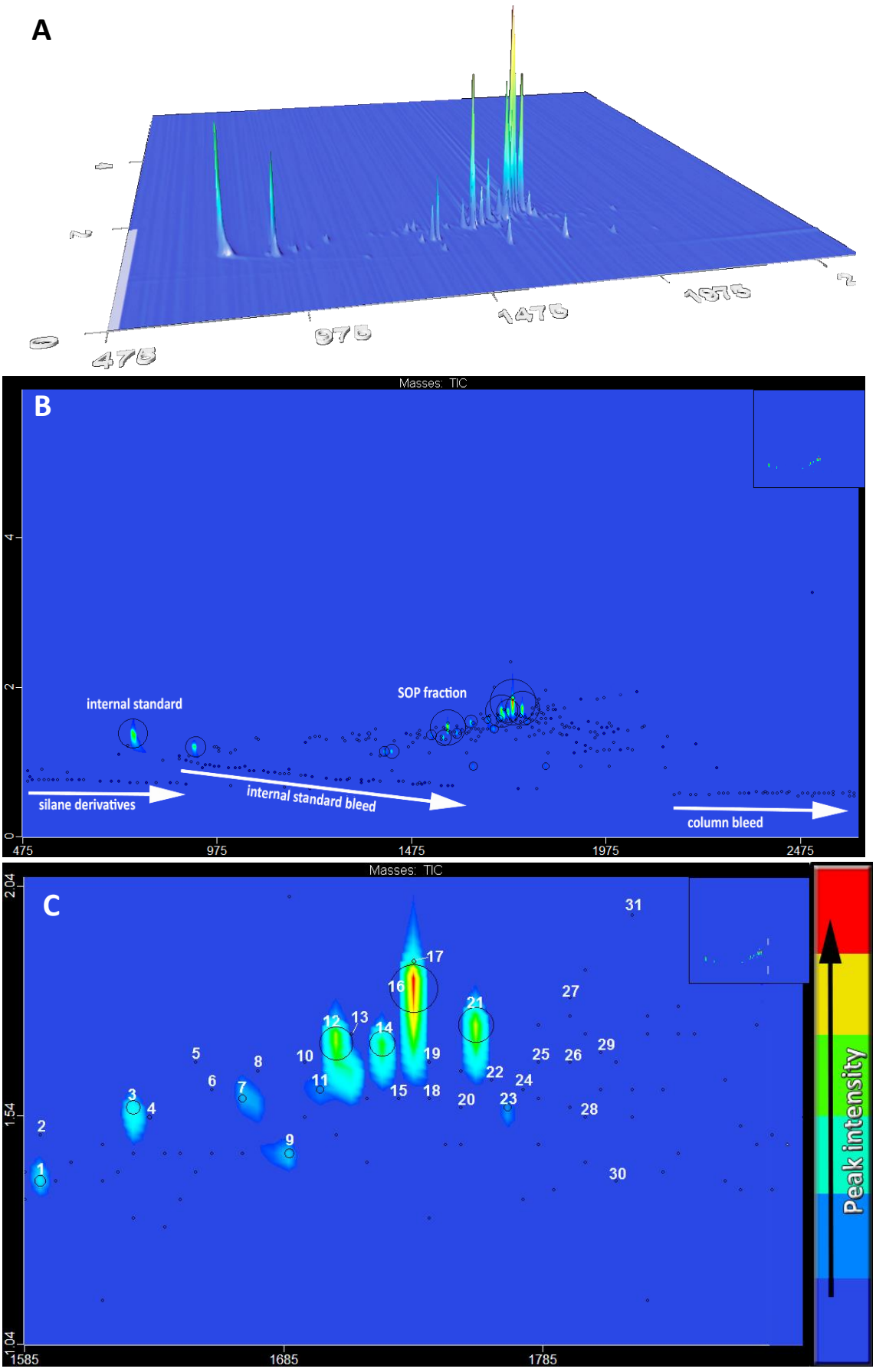

Figure 6-11. 3-D (A), 2-D plot (B) and detailed 2 D-plot (C) of the SOP fraction upon GCxGC-TOFMS analysis. X-axis= first-column separation (retention time: 475 – 2475 s). Y-axis= second-column separation (modulation every 6 s). Numbering: see **Table 6-3** for identification of volatiles.

Table 6-3. Compounds tentatively identified in SOP-fraction upon GCxGC-TOFMS analysis. Only compounds with a relative area % > 0.05% are given. N°= numbering in accordance with **Figure 6-11 C**. t_R 1(s)= first column retention time. t_R 2(s)= second column retention time.

N°	t_R1 (s)	t_R2 (s)	Peak area	Area %	Tentatively identified compound
	901	1.1	1323835	0.30	β-Pinene
	919	1.22	45224809	10.2	β-Myrcene
	967	1.22	252829	0.06	*meta*-Cymene
	979	1.16	1525806	0.34	Limonene
		1.2	270216	0.06	Oxygenated monoterpenoid (m/z 55, 71, 81, 96, 108, 110, 119, 139, 154)
	1021	1.18	484385	0.11	γ-Terpinene
	1081	1.32	2788750	0.63	Perillene
	1225	1.36	366181	0.08	Decanol
	1297	1.34	262714	0.06	Methyl ketone
	1303	1.3	267810	0.06	Ethyl ester
	1333	1.32	513984	0.12	Methyl ester
		1.38	3447465	0.77	Methyl ketone
	1363	1.4	3007041	0.67	Methyl 4-decenoate
	1369	1.12	887565	0.20	Hydrocarbon
	1381	1.32	338615	0.08	Methyl ester
		1.46	594828	0.13	Methyl geranate
	1471	1.38	1555870	0.35	Methyl ketone
	1489	1.4	428478	0.10	Unknown (m/z 55, 67, 82, 96, 124, 138)
	1495	1.38	269462	0.06	Unknown (m/z 59, 74, 82, 101, 124, 166)
	1507	1.34	229971	0.05	Unknown oxygenated sesquiterpenoid
	1525	1.38	13953563	3.13	β-Caryophyllene
	1549	1.36	338514	0.08	Methyl ketone
	1555	1.34	17609771	3.95	β-Farnesene
		1.48	2038940	0.46	Unknown oxygenated sesquiterpenoid (m/z 69, 81, 95, 109, 123, 138, 149, 191, 205, 220)
	1561	1.46	597169	0.13	Unknown (m/z 43, 54, 67, 81, 96, 110, 125, 138)
	1567	1.44	235273	0.05	Unknown (m/z 92)
		1.48	40941962	9.19	α-Humulene
	1573	1.42	314324	0.07	Unknown (m/z 43, 54, 67, 71, 79, 81, 93, 107, 109, 119, 121, 135, 147, 161, 204)
	1585	1.42	987691	0.22	Sesquiterpene hydrocarbon (m/z 55, 91, 93, 105, 119, 132, 145, 161, 189, 202, 204)
1	1591	1.4	8345251	1.87	2-Tridecanone
2		1.5	1672817	0.38	1,5,8,8,-tetramethyl-12-oxa-5-tricyclo[7.2.1.$0^{b,y}$]dodecene
	1597	1.4	1043508	0.23	Unknown (m/z 55, 69, 74, 84, 96, 110, 138, 161, 180)
	1603	1.44	3085053	0.69	Unknown sesquiterpene hydrocarbon (m/z 79, 93, 107, 121, 133, 149, 161, 189, 204)
	1615	1.4	829357	0.19	α-Muurolene
	1627	1.32	1173498	0.26	Unknown (m/z 57, 69, 83, 101, 112, 125, 143)
3		1.56	17433179	3.91	Unknown (m/z 69, 163, 173)
4	1633	1.54	3567449	0.80	*trans*-Calamenene
	1639	1.3	1901602	0.43	Unknown (m/z 57, 69, 83, 101, 112, 125, 143)
		1.46	506647	0.11	δ-Cadinene
5	1651	1.66	404389	0.09	13-nor-Z-caryophyllene-8-one
	1657	1.46	1966943	0.44	Unknown sesquiterpene (m/z 69, 79, 93, 105, 119, 134, 161, 187, 204)
6		1.6	915263	0.21	α-Calacorene
	1669	1.46	6231834	1.40	Unknown (m/z 93)
7		1.58	11164477	2.51	Isocaryophyllene epoxide A
8	1675	1.64	150533	0.06	β-Calacorene
9	1687	1.46	7408571	1.66	E-Dendrolasin
10	1693	1.66	73582	0.05	Caryophylla-4(12),8(13)-dien-5-one
11	1699	1.6	8279480	1.86	6(5→4)-Abeo-caryophyll-8(13)-en-5-al
	1705	1.5	1118474	0.25	2-Tetradecanone
12		1.7	59825737	13.4	Caryophyllene oxide
13	1711	1.72	249074	0.06	Unknown oxygenated sesquiterpenoid (m/z 107, 135, 218)
14	1723	1.7	36449907	8.18	Humulene epoxide I
15	1729	1.58	303171	0.07	Humulol
16	1735	1.82	56836546	12.8	Humulene epoxide II
17		1.88	6424268	1.44	Humulene allylic alcohol
18	1741	1.58	33431	0.06	1,11-di-*epi*-Cubenol
19		1.66	404750	0.09	α-Corocalene
	1753	1.48	1139360	0.26	Unknown (m/z 69, 175, 203)
20		1.56	111464	0.65	1-*epi*-Cubenol
		1.64	2637783	0.59	Humulenol II
21	1759	1.74	39123390	8.78	Humulene epoxide III (+ m/z 136: caryophylla-4(12),8(13)-diene-5-ol)
22	1765	1.62	524262	0.12	τ-Cadinol
23	1771	1.56	7056931	1.58	Unknown (m/z 79, 80, 81, 164, 122)
24	1777	1.6	1063550	0.24	14-hydroxy-β-Caryophyllene
	1783	1.48	3045309	0.68	Unknown (m/z 54, 67, 82, 96, 125, 166)
25		1.66	2278381	0.51	3Z-Caryophylla-3,8(13)-diene-5α-ol
	1795	1.56	229144	0.05	Unknown (m/z 69, 81, 177)
26		1.66	1291889	0.29	3Z-Caryophylla-3,8(13)-diene-5β-ol
27		1.76	2108972	0.47	Cadalene
28	1801	1.54	369332	0.08	10-hydroxy-*trans*-Calamenene
29		1.68	1180948	0.27	Humulene allylic alcohol
30	1813	1.4	793683	0.18	2-Pentadecanone
31	1819	1.98	43677	0.05	*nor*-Calamenen-10-one
			445613137	99.9	Total

From the studies of both Eyres *et al.*[114] and Nielsen[146], it appeared that the chromatographic region in which 14-hydroxy-β-caryophyllene and caryophylla-3,8(13)-diene-5β-ol elute also consists of other minor flavour-active compounds. However, due to the extreme complexity of this chromatographic region (many co-eluting OSs with highly similar spectra), they did not attempt to identify co-eluting compounds. Therefore, we investigated this chromatographic region into more detail. A 3D and 2D plot of this particular chromatographic region is given in **Figure 6-12**. Volatiles identified in this region are summarised in **Table 6-4**. From these results it can be concluded that besides the caryophyllene alcohols mentioned above, many other hop oil-derived constituents could be determined and tentatively identified in this particular region.

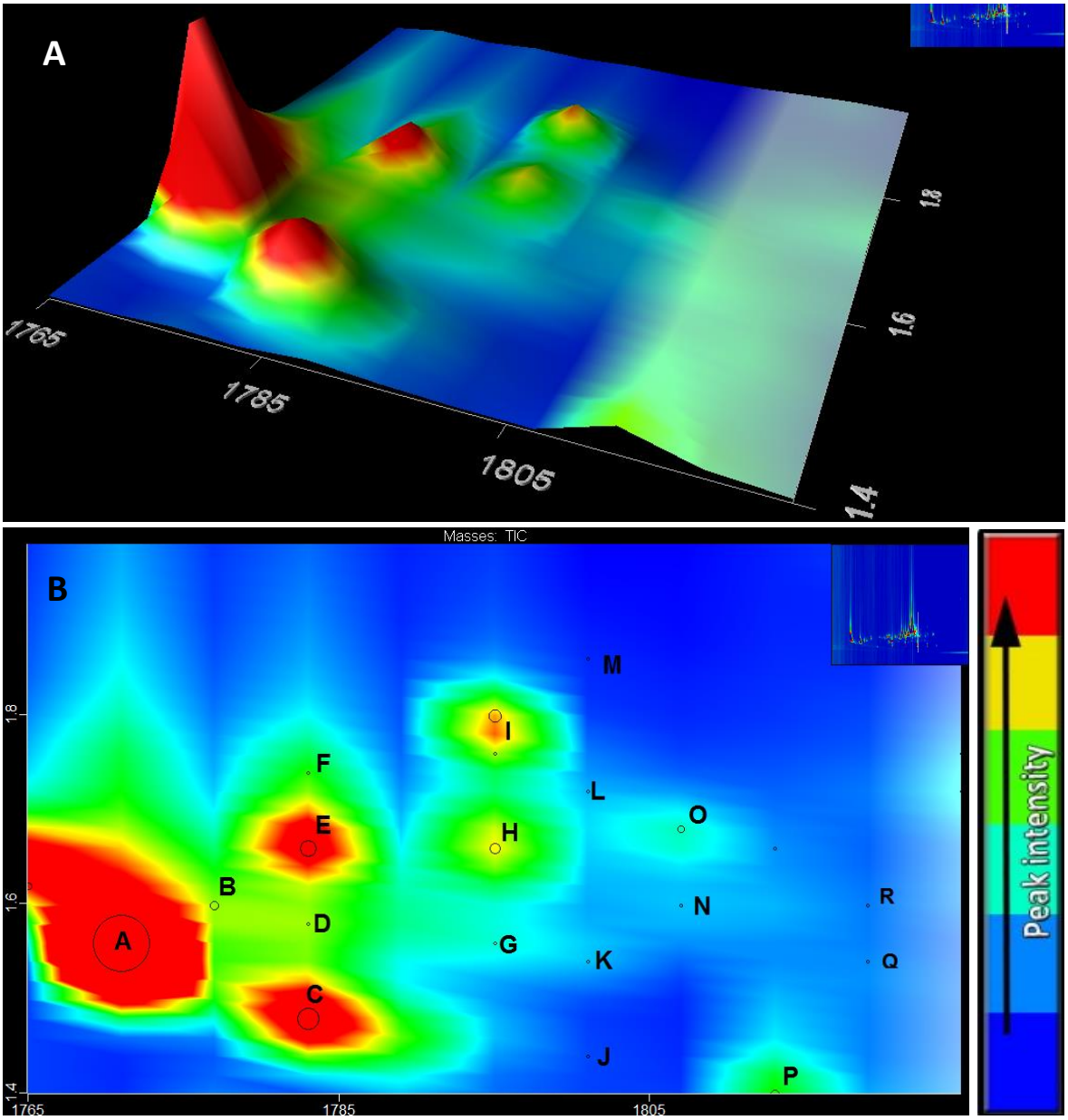

Figure 6-12. 3D plot (A) and 2D plot (B) of the chromatographic region of interest (starting from t_R 1=1765 and t_R 2= 1.4) of the SOP-fraction upon GCxGC-TOFMS analysis. Letters in acccordance to Table 6-4.

Table 6-4. Volatiles tentatively identified in chromatographic region (t_R1 1771 – t_R1 1819, see also Figure 6-12) of the SOP-fraction upon GCxGC-TOFMS analysis. Code in accordance with **Figure 6-12 B**. t_R 1 (s)= first column retention time. t_R 2(s)= second column retention time.

t_R 1 (s)	t_R 2 (s)	Peak area	Area %	Tentatively identified volatile	code
1771	1.56	7056931	36.49	Unknown (m/z 79, 80, 81, 164, 122)	A
1777	1.6	1063550	5.50	14-hydroxy-β-Caryophyllene	B
1783	1.48	3045309	15.75	6Z-Pentadecen-2-one	C
1783	1.58	184996	0.96	Unknown (m/z 69, 81)	D
1783	1.66	2278381	11.78	3Z-Caryophylla-3,8(13)-diene-5α-ol	E
1783	1.74	74948	0.39	Unknown (m/z 175)	F
1795	1.56	229144	1.18	2Z,6Z-Farnesol	G
1795	1.66	1291889	6.68	3Z-Caryophylla-3,8(13)-diene-5β-ol	H
1795	1.8	1193722	6.17	Cadalene	I
1801	1.44	72153	0.37	Unknown (m/z 85, 100)	J
1801	1.54	369332	1.91	10-hydroxy-*trans*-Calamenene	K
1801	1.72	55878	0.29	Unknown (m/z 93)	L
1801	1.86	12483	0.06	α-Cuparenone	M
1807	1.6	116554	0.60	Unknown (m/z 69, 81)	N
1807	1.68	1229470	6.36	Humulene allylic alcohol	O
1813	1.4	793683	4.10	2-Pentadecanone	P
1819	1.54	221181	1.14	2E,6Z-Farnesol	Q
1819	1.6	50442	0.26	α-*trans*-Bergamotol	R
		19340046	100.00	Total	

GCxGC-TOFMS analysis of beer aromatised with the SOP-fraction

Figure 6-13 shows the 3D and 2D plot of beer D-SOP upon GCxGC-TOFMS analysis. Using the first-column and second-column retention times of the volatiles of the SOP-fraction, the chromatogram of the beer aromatised with this fraction could easily be screened for the candidate key impact odour compounds of the SOP fraction, proposed in **Chapter 4** (*i.e.* humulene epoxide III, humulenol II, caryophylla-4(12),8(13)-diene-5-ol, 3Z-caryophylla-3,8(13)-diene-5α-ol, 14-hydroxy-β-caryophyllene and 3Z-caryophylla-3,8(13)-diene-5β-ol). The chromatographic region in which these compounds elute in the chromatogram of beer D-SOP is shown in **Figure 6-14**. Clearly, all the proposed potential flavour impact compounds were recovered in the beer whereas they could not be detected in the 'blank' beer (beer ISO; non-aromatised). Therefore, the SOPs mentioned above are proposed to contribute to the distinct 'spicy/herbal' notes.

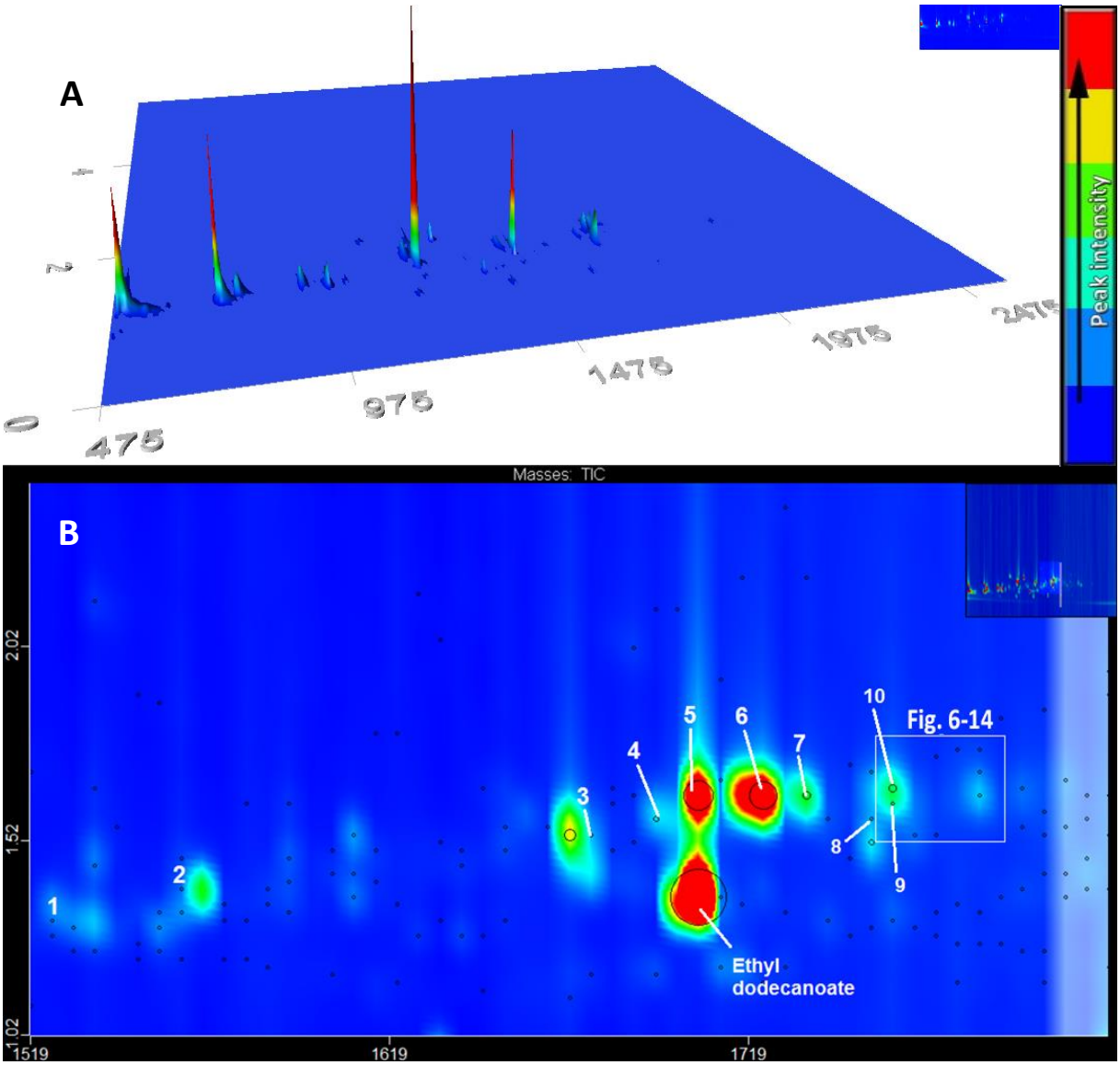

Figure 6-13. 3D plot (A), and 2D-plot (B) of the spicy region of beer D-SOP upon GCxGC-TOFMS analysis. X-axis= first-column separation. Y-axis= second-column separation. (1) β-caryophyllene, (2) α-humulene, (3) isocaryophyllene epoxide A, (4) humuladienone, (5) caryophyllene oxide, (6) humulene epoxide I, (7) humulene epoxide II, (8) humulenol II, (9) humulene epoxide III, (10) caryophylla-3(12),8(13)-diene-5-ol.

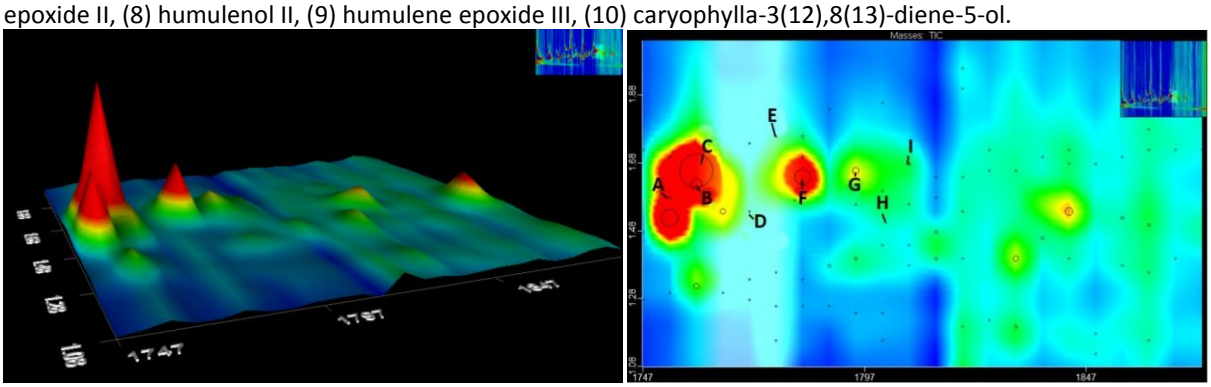

Figure 6-14. 3D plot (left) and 2D plot (right) of the chromatographic region indicated on Figure 6-13 (starting from t_R1 1747) upon GCxGC-TOFMS analysis of beer D-SOP. (A) humulenol II, (B) humulene epoxide III, (C) caryophylla-4(12),8(13)-diene-5-ol, (D) cubenol, (E) 14-hydroxy-β-caryophyllene, (F) 3Z-caryophylla-3,8(13)-diene-5α-ol, (G) 3Z-caryophylla-3,8(13)-diene-5β-ol, (H) 10-hydroxy-*trans*-calamenene, (I) humulene allylic alcohol.

6.4 Conclusions

In conclusion, we brewed one non-aromatised reference beer and 8 lager beers, aromatised with either Saaz hop pellets or hop oil (-derived fractions), thereby varying the hopping practice and time point of hop addition. Analysis of wort samples showed for the first time that *de novo* formation of sesquiterpene oxidation products (SOPs) occurs during wort boiling in real brewing practice when hop pellets are added 'early' in the process. During the whirlpool stage, net increases in the level of SOPs were not observed. On the other hand, several oxygenated monoterpenoids increased in levels as a consequence of whirlpool hopping and amongst these floral compounds, many volatiles were not detected in samples taken during the wort boiling process of the 'early' kettle hopped beer. Clearly, the hopping regime has a major impact on the composition of hop oil-derived volatiles detected in wort.

Although many compounds are lost during subsequent steps of the brewing process and hop oil levels that survive up to the final beer are at the ppb level, the differently hopped lager beers clearly expressed different flavour characteristics. In our brewing trials, addition of a portion of the hops at the onset of wort boiling and of another portion at the end, resulted in a highly appreciated and well-balanced beer with intense 'kettle hop' aroma. Apparently, this highly desired flavour characteristic seems to require a delicate balance between the 'spicy/herbal' and 'floral/citrus' bouquet. Although OS levels in the 'early' kettle hopped beer were relatively low, the flavour profile of the resulting beer was dominated by 'spicy/herbal' and 'woody' notes, confirming the general view that boiling of aroma hops is required to impart this typical flavour characterstic to beer.

Remarkably, the flavour profile of beer D-SOP (aromatised with SOPs) was similar to the flavour profile of the 'early' kettle hopped beer, indicating that the characteristic 'spicy/herbal' note can be mimicked by post-fermentation addition of SOPs, prepared off-line via oxidation of hop oil-derived SHCs. Post-fermentation addition of boiled total hop essential oil resulted in a beer that also showed 'spicy/herbal' notes and, moreover, this beer was scored higher for 'kettle hop' aroma, which may be related to the broader spectrum of hop oil volatiles in combination with increased SOP levels. Apparently, these two novel aromatisation techniques show potential for application in real brewing practice.

GCxGC-TOFMS analysis revealed the presence of humulene epoxide III, humulenol II, caryophylla-4(12),8(13)-diene-5-ol, 3Z-caryophylla-3,8(13)-diene-5α-ol, 14-hydroxy-β-caryophyllene and 3Z-caryophylla-3,8(13)-diene-5β-ol in beer D-SOP. This finding, together with the results obtained in **Chapter 4**, points to the above volatiles as key contributors to the 'spicy/herbal' bouqet of 'kettle hop' aroma.

GENERAL CONCLUSIONS AND PERSPECTIVES

The predominant influences on overall beer flavour are derived from malt components, yeast metabolism products, possibly adjuncts, and hop constituents. Hops are, from a quantitative perspective, only a minor ingredient when compared to the amount of malt used in brewing. Nevertheless, their impact on beer flavour is undeniable, which can be demonstrated by a statement of Verzele: "Not many people have had the opportunity to taste unhopped beer. This is a most revealing experience. The liquid reminds one of lemonade (but is sweeter and more acidic). The malt taste is not really pleasant and the alcohol flavour is decidedly there"[43]. To this respect, hops are unique due to their bittering potential (*i.e.* presence of hop α-acids) and, consequently, they have been used in brewing practice for centuries to overcome the sweet taste of unhopped beer. Beer bitterness has been studied extensively and, at present, the underlying chemistry is well understood. Next to bitterness, hops also play a pivotal role in beer flavour on account of the hop essential oil volatiles, imparting characteristic hop-derived aromas to the final beer. Many parameters have impact on the final hoppy aroma characteristics of beer. In particular the amount of added hops, point of time of hop addition and the hop variety are decisive in this regard. Although the application of advanced aromatisation techniques (*i.e.* use of hop oils and derived essences at post-fermentation) is growing in popularity and dry-hopping techniques are stimulated by the USA craft beer scene, traditional kettle hopping practices are still widely applied by brewers. Remarkably, despite decades of research, a fine hop-derived aroma, especially the so-called 'noble kettle hop' aroma (imparted by boiling of rather expensive (European) aroma hop varieties), remains however an elusive quality that is still poorly understood.

Over the years, researchers have been associating particular hop oil-derived volatiles to this highly desired flavour characteristic. Although many compounds have been proposed to contribute to 'kettle hop' aroma, linalool proves to be the only hop oil constituent that consistently appears above its flavour threshold in beer. This monoterpene alcohol is the major contributor to the 'floral' note, whereas monoterpene alcohols such as geraniol and citronellol may contribute to floral/citrusy scents via additive and/or synergetic effects. Research has been pointing to sesquiterpene oxidation products (SOPs) and their hydrolysed derivatives as contributors to 'spicy/herbal' aroma. Although at first none of these compounds seemed to exhibit 'spicy' or 'hoppy' aroma, recent investigations indicate particular flavour-active oxygenated sesquiterpenoids (OSs). Moreover, OS fractions isolated from total hop oil impart 'spicy' flavour characteristics upon addition to beer and sensory evaluation, suggesting that these compounds may jointly contribute to the 'spicy' impression of 'kettle hop' flavour. It is common knowledge that SOPs are formed during drying, kilning

and storage of hops and some brewers intentionally subject their hops to mild ageing to increase 'kettle hop' intensity in their beers. Although it has been proposed by some researchers that similar oxidation reactions may also occur during kettle boiling, opinions regarding this issue are controversial. Clearly, scientifically based insights into the development of 'kettle hop' aroma are fragmentary and, therefore, form the main purpose of this PhD study.

In first instance, a literature overview on hops and hop essential oil in particular, hop aroma, modifications of hop oil volatiles during hop storage, wort boiling and fermentation, and, finally, on hoppy aroma in beer was given.

For the practical part, fundamental insights were obtained in aqueous solutions on a lab scale, upon which we gradually worked towards beer to verify if our results also apply in real brewing practice. The combination of a variety of analytical techniques, chemometrics and sensory techniques, allowed us to extract novel insights into 'kettle hop aroma'.

Changes in the analytical hop oil fingerprint upon boiling of varietal hop essential oil cv. Saaz were studied into detail in **Chapter 2**. Since previous investigations have proven that conclusions are only painly achieved when comparing hopped worts and beers, we approached this issue from a different perspective, *i.e.* via simplified lab scale boiling experiments in closed systems. Such boiling experiments with total hop essential oil and subsequent analytical characterization of both unboiled and boiled hop essential oils has not been performed before. HS-SPME-GC-MS analysis of unboiled and boiled hop oil samples and subsequent multivariate analysis (PCA, CA) clearly demonstrated clustering between unboiled and boiled samples, respectively, indicating differences in the hop oil-derived volatile profile between unboiled and boiled hop essential oil. Strong decreases in levels of terpene hydrocarbon were observed upon boiling, whereas a clear increase in the level of spicy compounds was demonstrated for the first time. The increase of these compounds, in particular of α-humulene and β-caryophyllene oxidation products (*i.e.* humuladienone, caryophyllene oxide, humulene epoxide I, humulene epoxide II, humulene epoxide III, humulol, caryophylla-4(12),8(13)-diene-5-ol, caryophylla-3,8(13)-diene-5β-ol and, humulene allylic alcohol), suggests oxidation of sesquiterpene hydrocarbons (SHCs). Moreover, various hop oil-derived volatiles were exclusively detected in boiled samples, and the number of these volatiles appeared to increase with increasing hop oil concentration. The observed changes upon boiling, point to *de novo* generation of volatiles and might play an important role in the development of 'kettle hop' aroma since non-aromatised iso-α-acid bittered lager beers spiked with boiled hop essential oil clearly showed 'spicy' and 'hoppy' flavour characteristics.

To focus the search for individual compounds imparting 'spicy/hoppy' flavour impressions of boiled hop oil, and to facilitate separation, detection and identification, hop volatiles from unboiled and boiled hop oil were fractionated according to their polarity using Solid Phase Extraction (SPE) (**Chapter 3**). Preliminary sensory evaluations of the boiled hop oil fractions indicated the fractions eluting with 60%, 70% and 80% ethanol as the most promising ones regarding 'kettle hop' aroma. Therefore, these fractions were subjected to comprehensive profiling via HS-SPME-GC-MS. When comparing the SPE fractions of unboiled and boiled hop oil, additional volatiles characteristic for boiled hop essential oil could be determined, further confirming *de novo* generation of volatiles upon boiling. Sensory evaluations of the hop oil fractions in non-aromatised iso-α-acid bittered lager beer demonstrated that the boiled hop oil fractions eluting with 70% and 80% ethanol, which contained high levels of 'spicy' compounds, imparted 'spicy' and 'hoppy' flavours. α-Humulene-derived epoxides and both α-humulene and β-caryophyllene derived alcohols were frequently detected in flavour-active zones upon GC-O analysis of these fractions. Application of SPE fractionation and HS-SPME-GC-MS analysis on commercial kettle hopped lager beers allowed us to present a detailed fingerprint of the OSs present in beer for the first time. Moreover, the presence of iso-korajol, 4S-dihydrocaryophyllene-5-one, 6(5→4)-abeo-8,12-cyclo-caryophyllan-5-al and 6(5→4)-abeo-caryophyll-8(13)-en-5-al in lager beer was reported for the first time. Many compounds formed *de novo* upon lab scale boiling of hop oil (cv. Saaz) were detected in the commercial beers, indicating that the lab scale boiling experiments may be relevant to real brewing practice. By combination of two novel approaches (*i.e*. fractionation boiled hop oil and GC-O vs. fractionation hop oil-derived volatiles in beer and GC-O), we ware able to determine α-humulene and β-caryophyllene oxidation products in flavour-active zones of boiled hop essential oil as well as in a commercial kettle hopped beer.

Since the above results pointed to the relevance of SOPs for 'kettle hop' aroma, we further focussed on this class of hop oil volatiles in **Chapter 4**. The novel aspect of this chapter comprises enrichment of sesquiterpene hydrocarbons (SHCs) from total hop essential oil cv. Saaz using SPE and subsequent lab scale boiling to unambiguously prove oxidation of SHCs. Moreover, SPE isolation of the SOPs formed *de novo* and addition of this particular fraction to non-aromatised iso-α-acid bittered lager beer demonstrated a cause-and-effect relationship between these constituents and 'spicy/kettle hop' flavour, which has not been proven before. Via GC-O analysis of the oxidation product fraction, two intervals with significant odour-activity were found. Humulene epoxide III, humulenol II, caryophylla-4(12),8(13)-diene-5α/β-ol, (3Z)-caryophylla-3,8(13)-diene-5α-ol, 14-hydroxy-β-caryophyllene and (3Z)-caryophylla-3,8(13)-diene-5β-ol were identified in these intervals and because (except for humulene epoxide III) all of these oxidation products were also found in flavour-active zones of a commercial kettle hopped lager beer, we propose these volatiles as

candidate key impact compounds for (the 'spicy/herbal/woody' aspect of) 'kettle hop' aroma. Moreover, we perforemd GC-O on both a mixture of reference OSs and *Betula* buds to confirm flavour-activity of caryophyllene allylic alcohols.

From the results obtained in **Chapter 2**, it appeared that the hop oil concentration has a significant impact on changes in the hop oil volatile profile during boiling. Therefore, in **Chapter 5**, this concentration effect was further explored. Boiling experiments with increasing hop oil concentrations as performed in this PhD have not been reported before. As a consequence, a positive correlation between the initial hop oil concentration and formation of SOPs during boiling in model solutions was proven for the first time, which might find interesting applications in brewing practice. Boiling experiments in wort confirmed these results.

Varietal aspects were investigated through boiling experiments with hop essential oil cv. Saaz, cv. Hallertau Tradition, cv. Perle, and cv. Magnum in wort. It was concluded that a large series of α-humulene and β-caryophyllene oxidation products formed *de novo* upon boiling are chemically identical for the different investigated hop varieties. Differences in flavouring potential among varieties may therefore be attributed to the intrinsic hop oil composition (*e.g.* hop varieties containing high α-humulene, β-caryophyllene and initial OS levels have more potential to deliver significant levels of SOPs to the wort and, finally, to the beer). Indeed, many brewers use traditional European aroma hop varieties, which contain high humulene levels and oxidise at a fairly rate, to impart 'kettle hop' aroma to their beers.

Furthermore, by applying the novel approach of boiling of a SHC fraction cv. Super Pride, we were able to investigate whether cadinols and related compounds are formed by oxidation or not. We were not able to detect selinenols and related compounds (*i.e.* cadinols, muurolols, eudesmols) upon boiling of this selinene-rich SHC fraction, which supports the hypothesis that these compounds are biosynthesised by the hop plant.

In the final experimental chapter (**Chapter 6**), scientific insights obtained from lab scale boiling experiments were verified in real brewing practice. Therefore, one non-aromatised reference beer and eight lager beers, aromatised with either Saaz hop pellets or hop oil (-derived fractions) were brewed in our pilot installation, thereby varying the hopping practice (conventional hopping vs. advanced aromatisation) and point of hop addition ('early', 'late', 'whirlpool' and 'post-fermentation' hopping, respectively).

Analysis of wort samples proved for the first time that *de novo* formation of SOPs also occurs during wort boiling in real brewing practice when hop pellets are added 'early' in the process. As opposed to the opinion of many brewers, 'early' hopping clearly imparted hop-derived aroma to the final beer. Furthermore, net increases in levels of SOPs upon whirlpool

hopping were not observed, and, a series of oxygenated monoterpenoids was found to be specific for hopping at this stage.

Clearly, the hopping regime has a major impact on the composition of hop oil volatiles detected in wort and, although the amount of these volatiles that survives up to the final beer is at the ppb level, these differences came to clear expression upon sensory evaluation of the final beers. Although 'early' hop addition and thus vigourous boiling of aroma hops clearly imparted the 'spicy/herbal' aspect of 'kettle hop' flavour to beer, a delicate balance with a 'floral/citrus' bouquet, obtained via 'late' hop addition, appeared to be essential to obtain the broad complex spectrum of 'kettle hop' flavour. Interestingly, the flavour of the beer aromatised with the SOP fraction (advanced post-fermentation aromatisation) approximated the flavour of the conventionally 'early' kettle hopped beer.

Using comprehensive multidimensional gas chromatography-mass spectrometry (GCxGC-TOFMS), the candidate key impact compounds proposed in **Chapter 4** could be identified in both the SOP fraction in beer with added SOP fraction. Moreover, addition of this oxidation product fraction positively influenced the bitterness perception (both intensity and quality) and significantly increased mouthfeel aspects. These effects were also observed upon post-fermentation addition of boiled total hop essential oil. In this case, besides distinct 'spicy/herbal' notes, 'floral' and 'citrusy' impressions were also detected and, therefore, the beer received a high score for 'kettle hop' aroma. It was concluded that the hop flavour profile of this beer is highly comparable to a classic kettle hopped traditional Pilsner-type lager beer. These novel aromatisation techniques (*i.e*. post-fermentation aromatisation with boiled hop oil or the SOP fraction) thus provide interesting flavour characteristics to beer and are therefore promising to mimic 'kettle hop' flavour.

Basically, the results from this PhD study provide essential **scientific insights towards a better understanding of 'kettle hop' aroma**:

- The boiling process generates new hop oil-derived volatiles, both during lab scale boiling experiments and in real brewing practice.
- Increases in levels of α-humulene and β-caryophyllene-derived alcohols (*e.g.* humulenol II, caryophylla-4(12),8(13)-diene-5α/β-ol, (3Z)-caryophylla-3,8(13)-diene-5α-ol, 14-hydroxy-β-caryophyllene and (3Z)-caryophylla-3,8(13)-diene-5β-ol) during boiling may play a key role into development of 'kettle hop' aroma.
- Since most volatiles formed *de novo* upon boiling are chemically identical for the different investigated varieties, the intrinsic hop oil composition (*i.e.* presence of volatiles specific for a particular variety and levels of oxygenated sesquiterpenoids (OSs), α-humulene and β-caryophyllene) is decisive for the potential of hops to impart 'kettle hop' aroma.

- Formation of sesquiterpene oxidation products (SOPs) during boiling can be positively influenced by increasing the initial hop oil concentration.
- An 'early' kettle hopped beer differentiates itself from a non-aromatised iso-α-acid bittered lager beer by its distinct 'spicy/herbal' impression, proving that not all hop oil volatiles are lost by stripping and that residual hop oil-derived constituents impact beer flavour.
- It was not possible to indicate individual hop oil-derived constituents expressing 'kettle hop' flavour characteristics. 'Kettle hop' aroma is proposed to be the result of a delicate balance between odour-active OSs, imparting 'spicy/herbal' impressions, and volatiles imparting 'floral/citrusy' notes.
- Post-fermentation aromatisation of beer with SOPs results in 'spicy/herbal' flavour, whereas the broader spectrum of 'kettle hop' aroma can be obtained by post-fermentation addition of boiled total hop essential oil. These new hopping technologies are of interest for brewing practice since post-fermentation addition may result into less losses (and potentially cost-reduction) and, a more consistent aroma.

The preparation of hop products specifically designed to impart hoppy aroma to beer is an important acivity of the hop processing industry. Over the last couple of decades, multiple hop oil preparations, like single variety total hop essential oils and essences with specific flavour notes, such as distinct floral, citrusy, spicy, herbal, fruity, and woody top notes, have been developed. In general, the terpene hydrocarbon fraction does not show attractive flavour characteristic and is considered waste material. A highly interesting application resulting from this PhD might be to oxidise the SHC fraction and to upscale the SPE technology to isolate the formed oxidation products. The resulting SOP fraction could then be applied to introduce spicy/herbal notes and to increase bitterness perception and fullness of beer. In particular, addition of this fraction to a beer aromatised via late hopping or by a floral hop essence could significantly increase 'kettle hop' aroma flavour characteristics. Also addition of boiled hop oil may find interesting brewery applications, as 'kettle hop' aroma characteristics can be imparted at the post-fermentation stage, resulting in a higher utilisation of hop oil volatiles compared to kettle additions. Moreover, post-fermenation aromatisation should result in an increased aroma consistency, which is an essential quality criterion of beer.

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APPENDIX

Appendix A. Composition of the SPE-fraction eluting with 60% EtOH, derived upon SPE fractionation of unboiled (U) and boiled (B) hop essential oil (cv. Saaz).

n°= number of the compound, RI= calculated retention index (RTX-1 capillary column), X= average (n=4), S.D.= standard deviation (n=4), R(%)= recovery of the volatile in B60 vs U60, expressed as %, identification= on the basis of mass spectrum (MS), retention index (RI) and reference compounds (RC). N= only detected in the boiled samples. - = no peak area given (co-elution and peak area negligible).

			Normalised peak area				Relative peak area (%)					
			U60		B60		U60		B60			
n°	**volatile**	**RI**	**X**	**S.D.**	**X**	**S.D.**	**X**	**S.D.**	**X**	**S.D.**	**R (%)**	**Identification**
1	2-METHYLBUTYL 2-METHYLPROPANOATE	1004	0.31	0.03	0.42	0.08	0.05	0.01	0.08	0.01	135	MS, RI
2	3-METHYLBUTYL 2-METHYLPROPANOATE / METHYL HEPTANOATE / UNKNOWN (m/z 82, 111, 127, 142)	1009	1.67	0.26	1.16	0.24	0.27	0.06	0.22	0.04	69	RC, MS, RI/RC, MS, RI/-
3	METHYL 2-METHYLHEPTANOATE	1048	1.71	0.33	1.94	0.32	0.27	0.04	0.38	0.05	113	MS, RI
4	METHYL 6-METHYLHEPTANOATE	1071	-	-	-	-	-	-	-	-	-	MS, RI
5	METHYL OCTANOATE / METHYL 2,6-DIMETHYLHEPTANOATE	1110	1.43	0.22	1.55	0.36	0.23	0.03	0.30	0.03	108	RC, MS, RI/MS, RI
6	METHYL 2-METHYLOCTANOATE	1148	0.22	0.05	0.27	0.08	0.03	0.01	0.05	0.01	119	MS, RI
7	METHYL 3-NONENOATE	1194	9.47	1.61	10.4	1.49	1.48	0.11	2.01	0.23	109	RC, MS, RI
8	METHYL NONANOATE	1208	5.33	0.88	3.62	0.87	0.83	0.06	0.69	0.05	68	RC, MS, RI
9	ETHYL NONANOATE	1246	3.66	0.54	2.00	0.37	0.57	0.04	0.39	0.05	55	RC, MS, RI
10	METHYL 4,6-DIMETHYLOCTANOATE	1264	1.72	0.29	0.79	0.22	0.27	0.02	0.15	0.02	46	MS, RI
11	METHYL *trans*-4-DECENOATE	1292	88.2	19.5	56.4	14.0	13.7	0.57	10.8	0.82	64	MS, RI
12	METHYL 3,6-DODECADIENOATE	1484	21.6	5.24	5.58	1.58	3.34	0.15	1.06	0.16	26	MS, RI
	TOTAL ESTERS		**135**	**28.3**	**84.1**	**18.8**	**21.0**	**0.66**	**16.1**	**0.75**	**62**	
13	2-NONANONE	1071	7.65	1.16	7.64	1.43	1.20	0.14	1.48	0.23	100	RC, MS, RI
14	NONANAL	1084	4.32	0.81	1.43	0.32	0.68	0.07	0.28	0.05	33	RC, MS, RI
15	2-DECANONE	1173	25.6	4.15	29.0	5.10	4.01	0.40	5.60	0.58	113	RC, MS, RI
16	DECANAL	1188	3.07	0.45	2.85	0.54	0.48	0.05	0.55	0.06	93	RC, MS, RI
17	3,4-DIMETHYL 2-HEXANONE	1228	0.25	0.07	0.16	0.05	0.04	0.01	0.03	0.00	64	MS, RI
18	UNIDENTIFIED METHYL KETONE	1238	19.9	3.94	17.1	4.02	3.09	0.21	3.26	0.12	86	MS
19	5-UNDECEN-2-ONE	1255	29.8	5.66	32.4	6.03	4.65	0.39	6.24	0.57	109	MS, RI
20	2-UNDECANONE	1275	110	23.4	84.8	21.6	17.0	0.91	16.2	1.00	77	RC, MS, RI
21	2-DODECANONE	1374	13.0	3.49	2.85	1.47	1.99	0.11	0.52	0.17	22	RC, MS, RI
22	5-TRIDECEN-2-ONE	1444	10.7	3.06	3.88	1.29	1.64	0.10	0.73	0.12	36	MS, RI
23	2-TRIDECANONE	1473	14.8	4.23	1.14	0.33	2.27	0.30	0.22	0.04	8	RC, MS, RI
	TOTAL ALIPHATIC CARBONYL COMPOUNDS		**239**	**49.1**	**183**	**39.7**	**37.1**	**1.75**	**35.0**	**0.50**	**77**	
24	DODECANOL	1455	11.9	3.48	13.4	1.74	1.82	0.17	2.62	0.43	113	RC, MS, RI
	TOTAL ALIPHATIC ALCOHOLS		**11.9**	**3.48**	**13.4**	**1.74**	**1.82**	**0.17**	**2.62**	**0.43**	**113**	
25	β-PINENE	<1000	0.35	0.03	0.00	0.00	0.06	0.01	0.00	0.00	0	RC, MS, RI

Appendix A continued

			Normalised peak area				Relative peak area (%)					
			U60		B60		U60		B60			
n°	volatile	RI	X	S.D.	X	S.D.	X	S.D.	X	S.D.	R (%)	Identification
26	β-MYRCENE	<1000	27.8	4.31	2.44	0.39	4.39	0.74	0.48	0.11	9	RC, MS, RI
27	β-PHELLANDRENE	1021	0.22	0.06	0.21	0.02	0.04	0.01	0.04	0.01	98	MS, RI
	TOTAL MONOTERPENE HYDROCARBONS		**28.4**	**4.32**	**2.66**	**0.38**	**4.48**	**0.76**	**0.52**	**0.12**	**9**	
28	UNKNOWN MONOTERPENOID (m/z 67, 71, 79, 81, 93, 107, 122)	1062	0.00	0.00	0.20	0.06	0.00	0.00	0.04	0.02	N	MS
29	LINALOOL	1088	-	-	-	-	-	-	-	-	-	RC, MS, RI
30	LINALYL ETHYL ETHER	1164	0.00	0.00	0.18	0.01	0.00	0.00	0.04	0.01	N	MS, RI
31	METHYL GERANATE	1302	7.93	1.64	7.10	1.39	1.23	0.12	1.36	0.10	90	RC, MS, RI
	TOTAL OXYGENATED MONOTERPENOIDS AND DERIVATIVES		**7.93**	**1.64**	**7.49**	**1.35**	**1.23**	**0.12**	**1.44**	**0.10**	**94**	
32	β-CARYOPHYLLENE	1407	1.62	0.70	0.78	0.27	0.25	0.10	0.15	0.02	48	RC, MS, RI
33	α-HUMULENE	1438	9.99	4.15	3.88	0.73	1.53	0.57	0.75	0.05	39	RC, MS, RI
34	β-FARNESENE	1441	2.64	1.02	3.91	0.87	0.41	0.15	0.75	0.09	148	RC, MS, RI
35	SESQUITERPENE HYDROCARBON (m/z 93, 109, 119, 145, 204)	1464	1.82	0.90	2.92	0.39	0.27	0.08	0.57	0.09	161	MS
36	β-CURCUMENE	1496	1.70	0.63	0.00	0.00	0.26	0.05	0.00	0.00	0	MS, RI
	TOTAL SESQUITERPENE HYDROCARBONS		**17.8**	**6.75**	**11.5**	**2.11**	**2.72**	**0.82**	**2.21**	**0.17**	**65**	
37	UNKNOWN OYGENATED SESQUITERPENOID (m/z 55, 69, 81, 95, 109, 123, 138, 149, 205, 220)	1433	1.08	0.19	0.80	0.11	0.17	0.02	0.16	0.02	74	MS
38	UNKNOWN OXYGENATED SESQUITERPENOID (m/z 55, 69, 81, 95, 109, 123, 138, 149, 205, 220)	1468	1.03	0.36	1.41	0.64	0.16	0.02	0.28	0.14	136	MS
39	$\Delta^{2,3}$-5α,8α-EPOXY-CARYOPHYLLANE	1494	0.00	0.00	1.56	0.51	0.00	0.00	0.29	0.03	N	MS, RI
40	4S-DIHYDROCARYOPHYLLENE-5-ONE	1524	0.49	0.16	4.16	1.91	0.07	0.01	0.77	0.19	857	MS, RI
41	UNKNOWN OXYGENATED SESQUITERPENOID (m/z 93, 107, 121, 205, 220)	1533	0.00	0.00	1.39	0.47	0.00	0.00	0.26	0.04	N	MS
42	HUMULADIENONE / CARYOLAN-1-OL	1544	5.19	1.53	7.09	3.01	0.80	0.10	1.31	0.29	136	MS, RI/MS, RI
43	6(5→4)-ABEO-CARYOPHYLL-8(13)-EN-5-AL	1550	4.46	1.09	2.54	0.46	0.69	0.02	0.49	0.03	57	MS, RI
44	CARYOPHYLLENE OXIDE	1554	2.12	0.70	0.00	0.00	0.32	0.05	0.00	0.00	0	RC, MS, RI
45	CLOVENOL	1556	1.27	0.44	0.46	0.22	0.19	0.04	0.08	0.03	36	MS, RI
46	GLEENOL / UNKOWN OXYGENATED SESQUITERPENOID (m/z 107, 135)	1561	1.17	0.36	0.84	0.37	0.18	0.03	0.16	0.04	72	MS, RI/MS
47	HUMULENE EPOXIDE I	1569	7.10	2.29	33.2	9.73	1.08	0.10	6.27	0.55	467	MS, RI
48	HUMULOL	1574	1.00	0.45	15.4	3.03	0.15	0.04	2.95	0.12	1536	MS, RI
49	HUMULENE EPOXIDE II	1579	1.97	0.80	1.18	0.18	0.30	0.10	0.23	0.05	60	MS, RI
50	HUMULENE ALLYLIC ALCOHOL	1587	1.06	0.31	2.70	0.86	0.16	0.03	0.51	0.08	255	MS, RI
51	1,10-DI-EPI-CUBENOL	1590	2.06	0.82	0.94	0.14	0.31	0.05	0.18	0.03	46	MS, RI
52	HUMULENE EPOXIDE III	1600	1.93	0.75	3.14	0.69	0.29	0.05	0.60	0.05	162	MS, RI

Appendix A continued

			Normalised peak area				Relative peak area (%)					
			U60		B60		U60		B60			
n°	volatile	RI	X	S.D.	X	S.D.	X	S.D.	X	S.D.	R (%)	Identification
53	HUMULENOL II	1603	8.89	2.30	35.0	8.84	1.37	0.08	6.67	0.42	394	MS, RI
54	CARYOHYLLA-4(12),8(13)-DIENE-5α/β-OL	1606	3.56	1.29	4.46	0.20	0.54	0.07	0.88	0.20	125	MS, RI
55	τ-CADINOL / τ-MUUROLOL	1612	6.72	2.45	3.64	0.59	1.02	0.14	0.71	0.16	54	MS, RI/MS, RI
56	δ-CADINOL	1617	1.40	0.81	1.05	0.24	0.21	0.07	0.20	0.02	75	MS, RI
57	α-CADINOL	1625	1.53	0.55	1.18	0.32	0.23	0.04	0.22	0.02	77	MS, RI
58	3Z-CARYOPHYLLA-3,8(13)-DIENE-5α-OL	1627	4.71	1.39	8.03	1.93	0.72	0.05	1.53	0.11	170	MS, RI
59	3Z-CARYOPHYLLA-3,8(13)-DIENE-5β-OL	1641	2.47	0.86	7.32	1.09	0.38	0.04	1.42	0.12	296	MS, RI
60	β-BISABOLOL	1645	3.03	1.38	0.00	0.00	0.45	0.12	0.00	0.00	0	MS, RI
61	HUMULENE ALLYLIC ALCOHOL	1648	3.73	1.33	4.41	0.22	0.57	0.10	0.87	0.15	118	MS, RI
	TOTAL OXYGENATED SESQUITERPENOIDS		**68.0**	**21.8**	**142**	**33.2**	**10.4**	**1.00**	**27.1**	**0.69**	**209**	
62	2H-2-ETHENYL TETRAHYDRO-2,6,6-TRIMETHYL-PYRAN	<1000	0.00	0.00	0.21	0.03	0.00	0.00	0.04	0.01	N	MS
63	PERILLENE	1088	3.99	0.71	2.96	0.77	0.62	0.07	0.57	0.11	74	MS, RI
64	E-DENDROLASIN	1550	-	-	-	-	-	-	-	-	-	MS, RI
	TOTAL PYRANS AND FURANS		**3.99**	**0.71**	**3.17**	**0.79**	**0.62**	**0.07**	**0.61**	**0.11**	**80**	
65	UNKNOWN	1039	0.07	0.03	0.07	0.03	0.01	0.00	0.01	0.00	103	
66	UNKNOWN	1159	0.68	0.13	0.43	0.12	0.11	0.01	0.08	0.02	63	
67	UNKNOWN	1187	0.52	0.10	0.00	0.00	0.08	0.02	0.00	0.00	0	
68	UNKNOWN (m/z 69, 100)	1204	1.16	0.17	1.21	0.14	0.18	0.02	0.24	0.04	105	
69	UNKNOWN	1266	2.19	0.27	2.26	0.35	0.35	0.06	0.44	0.02	103	
70	UNKNOWN (m/z 85, 150)	1294	38.4	8.46	41.0	6.59	5.95	0.39	7.91	0.57	107	
71	UNKNOWN	1308	0.57	0.14	0.00	0.00	0.09	0.02	0.00	0.00	0	
72	UNKNOWN	1311	0.41	0.10	0.00	0.00	0.06	0.02	0.00	0.00	0	
73	UNKNOWN	1350	1.02	0.29	0.57	0.25	0.16	0.02	0.11	0.02	56	
74	UNKNOWN	1361	0.27	0.09	0.15	0.02	0.04	0.01	0.03	0.00	56	
75	UNKNOWN	1364	1.13	0.27	0.63	0.17	0.17	0.02	0.12	0.02	56	
76	UNKNOWN	1388	0.35	0.09	0.18	0.05	0.05	0.01	0.03	0.01	51	
77	UNKNOWN (m/z 79, 80, 81, 93, 122, 136, 164)	1390	8.48	1.91	2.93	0.91	1.31	0.05	0.55	0.08	35	
78	UNKNOWN	1413	0.59	0.41	1.09	0.34	0.08	0.04	0.21	0.04	186	
79	UNKNOWN	1418	1.17	0.50	1.13	0.47	0.18	0.04	0.21	0.06	96	
80	UNKNOWN	1452	2.45	0.78	1.13	0.32	0.37	0.03	0.21	0.03	46	
81	UNKNOWN	1489	1.19	0.42	0.00	0.00	0.18	0.03	0.00	0.00	0	
82	UNKNOWN (m/z 79, 80, 81, 150)	1536	12.0	3.34	1.86	0.97	1.85	0.13	0.34	0.14	15	
83	UNKNOWN	1542	0.92	0.28	0.69	0.61	0.14	0.01	0.12	0.09	75	
84	UNKNOWN	1595	2.01	0.81	2.41	0.64	0.30	0.05	0.48	0.16	120	

Appendix A continued

			Normalised peak area				Relative peak area (%)					
			U60		B60		U60		B60			
n°	volatile	RI	X	S.D.	X	S.D.	X	S.D.	X	S.D.	R (%)	Identification
85	UNKNOWN (m/Z 79, 80, 81, 164, 179, 222)	1632	31.4	10.1	2.70	0.91	4.82	0.78	0.51	0.09	9	
86	UNKNOWN (m/z 79, 80, 81, 162)	1634	21.6	6.17	5.61	1.67	3.31	0.26	1.08	0.23	26	
87	UNKNOWN (m/z 93, 137)	1637	2.88	1.00	3.24	0.64	0.44	0.05	0.62	0.03	112	
88	UNKNOWN (m/z 145)	1648	-	-	-	-	-	-	-	-	-	
89	UNKNOWN	1657	1.00	0.56	2.04	0.07	0.15	0.05	0.40	0.09	204	
90	UNKNOWN	1659	1.42	0.48	3.47	0.25	0.22	0.03	0.68	0.10	244	
	TOTAL UNKNOWN VOLATILES		**134**	**35.1**	**74.8**	**12.8**	**20.6**	**0.91**	**14.4**	**0.54**	**56**	
	MONOTERPENE HYDROCARBONS		**28.4**	**4.32**	**2.66**	**0.38**	**4.48**	**0.76**	**0.52**	**0.12**	**9**	
	FLORAL FRACTION		**395**	**80.4**	**318**	**65.2**	**61.4**	**3.56**	**60.9**	**0.44**	**81**	
	SESQUITERPENE HYDROCARBONS		**17.8**	**6.75**	**11.5**	**2.11**	**2.72**	**0.82**	**2.21**	**0.17**	**65**	
	SPICY FRACTION		**205**	**61.3**	**190**	**41.4**	**31.4**	**2.73**	**36.4**	**0.48**	**93**	
	TOTAL OF HOP OIL-DERIVED COMPOUNDS		**646**	**146**	**522**	**108**	**100**	**0.00**	**100**	**0.00**	**81**	

Appendix B. Composition of the SPE-fraction eluting with 70% EtOH, derived upon SPE fractionation of unboiled (U) and boiled (B) hop essential oil (cv. Saaz).
n°= number of the compound, RI= retention index (RTX-1 capillary column), X= average (n=4), S.D.= standard deviation (n=4), R(%)= recovery of the volatile in B70 vs U70, expressed as %, identification= on the basis of mass spectrum (MS), retention index (RI) and reference compounds (RC). N= only detected in the boiled samples. - = no peak area given (co-elution and peak area negligible).

			Normalised peak area				Relative peak area (%)					
			U70		B70		U70		B70			
n°	volatile	RI	X	S.D.	X	S.D.	X	S.D.	X	S.D.	R (%)	Identification
1	METHYL 2-METHYLHEPTANOATE	1049	0.68	0.12	0.54	0.15	0.04	0.00	0.02	0.00	79	MS, RI
2	METHYL 6-METHYLHEPTANOATE	1072	2.31	0.25	1.50	0.48	0.13	0.02	0.05	0.01	65	MS, RI
3	METHYL OCTANOATE / METHYL 2,6-DIMETHYLHEPTANOATE	1110	1.18	0.17	1.17	0.40	0.06	0.00	0.04	0.01	100	RC, MS, RI/MS, RI
4	METHYL 2-METHYLOCTANOATE	1148	0.38	0.05	0.68	0.29	0.02	0.00	0.02	0.01	181	MS, RI
5	METHYL 4-METHYLOCTANOATE	1177	1.41	0.08	1.52	0.22	0.08	0.01	0.06	0.01	108	MS, RI
6	UNKNOWN ETHYL ESTER	1182	0.87	0.13	1.22	0.28	0.05	0.00	0.05	0.01	141	MS
7	METHYL 3-NONENOATE	1194	5.95	0.55	5.33	1.15	0.32	0.03	0.20	0.02	90	RC, MS, RI
8	METHYL NONANOATE	1208	12.2	1.66	15.0	3.16	0.66	0.03	0.55	0.04	123	RC, MS, RI
9	HEPTYL BUTANOATE	1235	0.16	0.06	0.22	0.04	0.01	0.00	0.01	0.00	137	MS, RI
10	ETHYL NONANOATE	1244	18.6	3.01	30.6	6.24	1.00	0.02	1.13	0.04	165	RC, MS, RI
11	METHYL 4,6-DIMETHYLOCTANOATE	1263	7.01	1.32	9.68	2.04	0.37	0.02	0.36	0.01	138	MS, RI
12	UNKNOWN ESTER (m/z 74, 87, 143)	1266	2.52	0.58	2.85	0.69	0.13	0.02	0.10	0.01	113	MS
13	UNKNOWN ESTER (m/Z 88, 101)	1280	5.50	0.88	9.96	2.56	0.29	0.01	0.36	0.02	181	MS
14	METHYL *trans*-4-DECENOATE	1293	230	33.8	280	53.1	12.3	0.47	10.4	0.45	122	MS, RI
15	METHYL DECANOATE	1308	6.10	1.49	7.80	1.49	0.32	0.04	0.29	0.02	128	RC, MS, RI
16	UNKNOWN ESTER	1324	0.45	0.02	0.65	0.26	0.02	0.00	0.02	0.01	144	MS
17	UNKNOWN ACETATE ESTER	1350	1.26	0.23	2.03	0.54	0.07	0.00	0.07	0.00	161	MS
18	ETHYL *cis*-4-DECENOATE	1361	1.20	0.22	4.17	1.28	0.06	0.00	0.15	0.02	347	MS, RI
19	METHYL 10-UNDECENOATE	1383	1.50	0.30	2.64	0.69	0.08	0.00	0.10	0.01	175	MS, RI
20	METHYL *trans*-3-DODECENOATE	1477	7.09	1.40	15.6	7.40	0.38	0.03	0.56	0.17	219	MS, RI
21	METHYL 3,6-DODECADIENOATE	1485	80.2	15.4	109	21.8	4.27	0.25	4.02	0.20	136	MS, RI
	TOTAL ESTERS		**386**	**61.2**	**502**	**101**	**20.7**	**0.73**	**18.6**	**0.38**	**130**	
22	NONANAL	1085	1.47	0.18	0.28	0.10	0.08	0.01	0.01	0.00	19	RC, MS, RI
23	2-DECANONE	1173	13.2	1.34	11.6	3.06	0.71	0.06	0.42	0.05	88	RC, MS, RI
24	DECANAL	1188	1.98	0.15	1.90	0.35	0.11	0.01	0.07	0.01	96	RC, MS, RI
25	3,4-DIMETHYL 2-HEXANONE	1228	0.29	0.03	0.00	0.00	0.02	0.00	0.00	0.00	0	MS, RI
26	UNKNOWN METHYL KETONE	1238	28.2	3.56	38.8	8.52	1.52	0.07	1.43	0.10	137	MS, RI
27	5-UNDECEN-2-ONE	1255	19.5	3.11	19.9	3.94	1.05	0.07	0.74	0.07	102	MS, RI
28	2-UNDECANONE	1276	199	26.9	300	61.4	10.7	0.51	11.1	0.50	151	RC, MS, RI
29	2-DODECANONE	1375	55.2	10.4	104	24.8	2.94	0.15	3.83	0.18	189	RC, MS, RI
30	UNIDENTIFIED KETONE	1437	4.54	1.40	10.1	3.09	0.24	0.05	0.37	0.06	222	MS

Appendix B continued

			Normalised peak area				Relative peak area (%)					
			U70		B70		U70		B70			
n°	volatile	RI	X	S.D.	X	S.D.	X	S.D.	X	S.D.	R (%)	Identification
31	*Cis*-5-TRIDECEN-2-ONE	1445	31.4	7.82	62.9	14.4	1.66	0.21	2.31	0.06	200	MS, RI
32	2-TRIDECANONE	1474	86.8	21.0	203	54.2	4.60	0.56	7.43	0.76	234	RC, MS, RI
33	UNKNOWN METHYL KETONE	1574	3.97	1.50	0.00	0.00	0.21	0.06	0.00	0.00	0	MS
34	6Z-PENTADECEN-2-ONE	1645	4.51	2.01	14.0	3.91	0.23	0.09	0.52	0.09	311	MS, RI
	TOTAL ALIPHATIC CARBONYL COMPOUNDS		**450**	**78.8**	**766**	**172**	**24.0**	**1.29**	**28.2**	**0.73**	**170**	
35	DODECANOL	1456	12.8	7.64	22.0	5.09	0.66	0.36	0.82	0.15	172	RC, MS, RI
	TOTAL ALIPHATIC ALCOHOLS		**12.8**	**7.64**	**22.0**	**5.09**	**0.66**	**0.36**	**0.82**	**0.15**	**172**	
36	β-PINENE	<1000	7.07	0.89	1.13	0.22	0.38	0.02	0.04	0.01	16	RC, MS, RI
37	β-MYRCENE	<1000	462	92.2	66.4	8.95	25.1	5.60	2.53	0.60	14	RC, MS, RI
38	P-CYMENE	1013	0.00	0.00	0.84	0.31	0.00	0.00	0.03	0.01	N	RC, MS, RI
39	LIMONENE	1021	2.21	0.31	1.45	0.42	0.12	0.01	0.05	0.01	66	RC, MS, RI
40	*Trans*-β-OCIMENE	1038	0.50	0.16	0.09	0.02	0.03	0.01	0.00	0.00	17	RC, MS, RI
41	TERPINOLENE	1079	0.59	0.08	0.76	0.06	0.03	0.00	0.03	0.01	128	RC, MS, RI
	TOTAL MONOTERPENE HYDROCARBONS		**472**	**92.9**	**70.7**	**9.59**	**25.6**	**5.63**	**2.69**	**0.62**	**15**	
42	LINALYL ETHYL ETHER	1163	0.00	0.00	5.54	0.81	0.00	0.00	0.21	0.02	N	MS, RI
43	TERPINYL ETHYL ETHER	1252	0.00	0.00	2.13	0.44	0.00	0.00	0.08	0.01	N	MS, RI
44	METHYL GERANATE	1303	10.1	1.54	10.1	1.66	0.54	0.01	0.38	0.02	100	RC, MS, RI
	TOTAL OXYGENATED MONOTERPENOIDS AND DERIVATIVES		**10.1**	**1.54**	**17.8**	**2.76**	**0.54**	**0.01**	**0.66**	**0.04**	**176**	
45	α-YLANGENE	1368	0.47	0.13	0.72	0.08	0.02	0.00	0.03	0.00	155	MS, RI
46	β-CARYOPHYLLENE	1407	9.55	3.41	2.75	0.59	0.50	0.13	0.10	0.01	29	RC, MS, RI
47	*Trans*-α-BERGAMOTENE	1426	0.35	0.08	1.53	0.68	0.02	0.00	0.06	0.02	436	MS, RI
48	α-HUMULENE	1439	56.2	17.9	18.4	4.19	2.96	0.66	0.68	0.09	33	RC, MS, RI
49	β-FARNESENE	1442	6.21	1.79	11.0	4.33	0.33	0.05	0.40	0.10	176	RC, MS, RI
50	UNKNOWN SESQUITERPENE HYDROCARBON	1459	1.71	0.75	0.00	0.00	0.09	0.03	0.00	0.00	0	MS
51	UNKNOWN SESQUITERPENE HYDROCARBON	1464	1.29	0.88	0.00	0.00	0.07	0.04	0.00	0.00	0	MS
52	UNKNOWN SESQUITERPENE HYDROCARBON	1497	2.35	0.20	0.00	0.00	0.13	0.02	0.00	0.00	0	MS
53	δ-CADINENE	1506	3.66	0.84	0.00	0.00	0.19	0.02	0.00	0.00	0	MS, RI
	TOTAL SESQUITERPENE HYDROCARBONS		**81.7**	**25.2**	**34.4**	**8.92**	**4.31**	**0.90**	**1.26**	**0.11**	**42**	
54	4α,8α-EPOXY-CARYOPHYLLANE	1402	0.00	0.00	0.41	0.14	0.00	0.00	0.02	0.00	N	MS, RI
55	4β,8β-EPOXY-CARYOPHYLLANE	1408	0.00	0.00	1.42	0.49	0.00	0.00	0.05	0.01	N	MS, RI
56	UNKNOWN OXYGENATED SESQUITERPENOID (m/z 137, 205, 220)	1428	0.00	0.00	1.56	0.41	0.00	0.00	0.06	0.01	N	MS

Appendix B continued

			Normalised peak area				Relative peak area (%)					
			U70		B70		U70		B70			
n°	volatile	RI	X	S.D.	X	S.D.	X	S.D.	X	S.D.	R (%)	Identification
57	UNKNOWN OXYGENATED SESQUITERPENOID (m/z 69, 81, 95, 109, 123, 138, 149, 205, 220)	1433	5.04	1.66	5.52	0.72	0.27	0.06	0.21	0.04	110	MS
58	1,5,8,8-TETRAMETHYL-12-OXA-5-TRICYCLO[7.2.1.0^{6,9}]DODECENE	1461	0.00	0.00	24.3	4.07	0.00	0.00	0.91	0.11	N	MS, RI
59	UNKNOWN (m/z 69, 81, 95, 109, 123, 138, 149, 205, 220)	1468	3.11	0.98	6.18	1.09	0.16	0.04	0.23	0.03	199	MS
60	$\Delta^{2,3}$-5α,8α-EPOXY-CARYOPHYLLANE	1496	0.00	0.00	23.4	8.16	0.00	0.00	0.84	0.13	N	MS, RI
61	UNKNOWN OXYGENATED SESQUITERPENOID (MW 220)	1502	0.00	0.00	2.27	0.69	0.00	0.00	0.08	0.02	N	MS
62	UNKNOWN OXYGENATED SESQUITERPENOID; SPECTRUM SIMILAR TO 4,8,11,11-TETRAMETHYL-8-TRICYCLO-[7.2.0.0^{2,5}]-UNDECEN-4-OL	1516	0.00	0.00	26.0	3.21	0.00	0.00	0.98	0.13	N	MS
63	4S-DIHYDROCARYOPHYLLENE-5-ONE	1524	0.00	0.00	36.0	11.7	0.00	0.00	1.30	0.16	N	MS, RI
64	4R-DIHYDROCARYOPHYLLENE-5-ONE	1528	0.00	0.00	2.43	0.86	0.00	0.00	0.09	0.01	N	MS, RI
65	MAALIOL / 6(5→4)-ABEO-CARYOPHYLL-7-EN-5-AL	1532	0.00	0.00	4.09	1.29	0.00	0.00	0.15	0.02	N	MS, RI/MS, RI
66	UNIDENTIFIED OXYGENATED SESQUITERPENOID(93, 107, 121, 205, 220)	1535	0.00	0.00	17.6	6.23	0.00	0.00	0.64	0.10	N	MS
67	HUMULADIENONE	1546	0.00	0.00	114	33.7	0.00	0.00	4.15	0.38	N	MS, RI
68	6(5→4)-ABEO-CARYOPHYLLAN-8(13)-EN-5-AL	1551	9.05	2.01	33.0	4.50	0.48	0.04	1.23	0.10	364	MS, RI
69	CARYOPHYLLENE OXIDE	1555	12.3	2.30	9.48	1.82	0.66	0.06	0.35	0.03	77	RC, MS, RI
70	CLOVENOL	1557	0.00	0.00	6.22	2.48	0.00	0.00	0.22	0.05	N	MS, RI
71	UNKNOWN (m/z 107, 135, 218)	1561	0.00	0.00	2.23	1.86	0.00	0.00	0.08	0.05	N	MS
72	GLEENOL	1564	2.75	0.68	3.13	0.91	0.15	0.02	0.11	0.02	114	MS, RI
73	HUMULENE EPOXIDE I	1572	16.8	3.55	159	48.5	0.90	0.08	5.77	0.64	942	MS, RI
74	HUMULOL	1575	0.00	0.00	39.5	9.07	0.00	0.00	1.45	0.08	N	MS, RI
75	HUMULENE EPOXIDE II	1580	24.7	2.93	10.1	1.89	1.33	0.10	0.38	0.06	41	MS, RI
76	HUMULENE ALLYLIC ALCOHOL	1588	1.06	0.31	14.0	4.10	0.06	0.01	0.51	0.05	1317	MS, RI
77	1,10-DI-EPI-CUBENOL	1591	3.27	0.77	9.79	2.53	0.17	0.02	0.36	0.02	299	MS, RI
78	JUNENOL	1592	0.85	0.31	0.00	0.00	0.04	0.01	0.00	0.00	0	MS, RI
79	HUMULENE EPOXIDE III	1601	7.80	1.89	50.5	9.76	0.41	0.04	1.87	0.07	647	MS, RI
80	HUMULENOL II	1604	22.8	5.82	54.2	16.3	1.20	0.16	1.97	0.22	238	MS, RI
81	CARYOPHYLLA-4(12),8(13)-DIENE-5α/β-OL	1607	6.07	1.49	10.6	1.89	0.32	0.04	0.39	0.04	174	MS, RI
82	τ-CADINOL / τ-MUUROLOL	1613	10.5	2.40	16.9	3.10	0.56	0.06	0.63	0.02	162	MS, RI/MS, RI
83	CUBENOL	1617	2.57	0.68	3.37	0.66	0.14	0.02	0.13	0.01	131	MS, RI
84	β-EUDESMOL	1619	0.25	0.07	1.07	0.38	0.01	0.00	0.04	0.01	430	MS, RI
85	SELIN-11-EN-4α-OL	1622	0.52	0.13	0.73	0.24	0.03	0.00	0.03	0.01	139	MS, RI
86	α-CADINOL	1625	2.66	0.64	5.19	1.40	0.14	0.01	0.19	0.01	195	MS, RI
87	3Z-CARYOPHYLLA-3,8(13)-DIENE-5α-OL	1628	9.25	2.76	20.4	4.41	0.49	0.09	0.75	0.08	221	MS, RI
88	3Z-CARYOPHYLLA-3,8(13)-DIENE-5β-OL	1644	5.31	1.89	11.4	4.15	0.28	0.07	0.41	0.07	215	MS, RI
89	β-BISABOLOL	1646	0.00	0.00	6.97	0.23	0.00	0.00	0.27	0.05	N	MS, RI
90	HUMULENE ALLYLIC ALCOHOL	1649	6.52	2.30	10.1	1.73	0.34	0.09	0.38	0.05	154	MS, RI

Appendix B continued

			Normalised peak area				Relative peak area (%)					
			U70		B70		U70		B70			
n°	volatile	RI	X	S.D.	X	S.D.	X	S.D.	X	S.D.	R (%)	Identification
91	EPI-α-BISABOLOL	1656	1.26	0.65	4.03	0.99	0.07	0.03	0.15	0.01	320	MS, RI
92	α-BISABOLOL	1659	1.51	1.04	3.40	0.29	0.08	0.05	0.13	0.02	225	MS, RI
	TOTAL OXYGENATED SESQUITERPENOIDS		**156**	**36.3**	**749**	**189**	**8.27**	**0.91**	**27.4**	**1.30**	**480**	
93	PERILLENE	1088	3.69	0.39	3.90	0.95	0.20	0.01	0.14	0.01	106	MS, RI
	TOTAL PYRANS AND FURANS		**3.69**	**0.39**	**3.90**	**0.95**	**0.20**	**0.01**	**0.14**	**0.01**	**106**	
94	UNKNOWN	1187	0.54	0.12	0.00	0.00	0.03	0.00	0.00	0.00	0	
95	UNKNOWN (m/z 69, 100)	1204	0.98	0.24	0.89	0.18	0.05	0.01	0.03	0.01	91	
96	UNKNOWN (m/z 43, 74, 81, 87, 99, 127)	1226	0.00	0.00	1.04	0.16	0.00	0.00	0.04	0.01	N	
97	UNKNOWN (m/z 85, 150)	1300	2.31	0.52	3.05	0.71	0.12	0.01	0.11	0.01	132	
98	UNKNOWN	1354	0.19	0.06	0.73	0.16	0.01	0.00	0.03	0.01	391	
99	UNKNOWN (m/z 43)	1363	2.08	0.34	3.57	0.78	0.11	0.01	0.13	0.01	171	
100	UNKNOWN	1388	1.22	0.29	1.74	0.35	0.06	0.01	0.06	0.00	142	
101	UNKNOWN (m/z 79, 80, 81, 93, 122, 136, 164)	1391	26.0	4.75	36.4	7.81	1.39	0.06	1.34	0.01	140	
102	UNKNOWN	1398	2.03	0.50	2.72	0.69	0.11	0.02	0.10	0.00	134	
103	UNKNOWN	1412	0.00	0.00	1.27	0.70	0.00	0.00	0.05	0.02	N	
104	UNKNOWN (m/z 97, 107, 122, 167, 220)	1418	1.83	0.82	5.73	2.00	0.10	0.04	0.21	0.03	313	
105	UNKNOWN (m/z 43, 55, 67, 81, 96, 110, 125, 138, 178)	1453	7.39	1.76	16.3	4.60	0.39	0.05	0.59	0.05	221	
106	UNKNOWN (m/z 79, 95, 108, 121)	1490	2.59	0.30	7.94	1.81	0.14	0.01	0.29	0.01	307	
107	UNKNOWN (m/z 69, 163)	1501	0.00	0.00	5.69	3.00	0.00	0.00	0.20	0.07	N	
108	UNKNOWN (m/z 79, 80, 81, 150, 157, 220)	1537	33.9	7.50	56.4	10.9	1.80	0.16	2.10	0.21	166	
109	UNKNOWN	1543	2.00	0.34	6.28	0.92	0.11	0.01	0.23	0.02	314	
110	UNKNOWN	1545	24.4	5.74	0.00	0.00	1.29	0.15	0.00	0.00	0	
111	UNKNOWN (m/z 69)	1584	0.00	0.00	6.84	3.50	0.00	0.00	0.24	0.08	N	
112	UNKNOWN	1587	0.92	0.17	0.00	0.00	0.05	0.01	0.00	0.00	0	
113	UNKNOWN	1596	1.44	0.87	2.71	0.81	0.07	0.04	0.10	0.02	189	
114	UNKNOWN (m/z 79, 80, 81, 164, 222)	1633	131	36.4	299	55.7	6.89	1.12	11.1	1.09	229	
115	UNKNOWN (m/z 79, 80, 81, 162, 220)	1635	44.4	8.04	63.2	12.9	2.37	0.12	2.35	0.36	142	
116	UNKNOWN	1638	5.34	1.87	9.75	2.04	0.28	0.07	0.36	0.04	183	
117	UNKNOWN	1641	3.22	1.62	0.00	0.00	0.17	0.07	0.00	0.00	0	
118	UNKNONWN (m/z 93, 137)	1642	0.00	0.00	8.19	1.30	0.00	0.00	0.31	0.03	N	
119	UNKNOWN	1651	2.03	0.86	3.52	0.66	0.11	0.04	0.13	0.03	173	
120	UNKNOWN (m/z 82, 85, 109, 121, 139)	1661	0.00	0.00	2.88	1.21	0.00	0.00	0.10	0.02	N	
	TOTAL UNKNOWN VOLATILES		**296**	**71.7**	**546**	**105**	**15.6**	**1.90**	**20.2**	**1.45**	**185**	

Appendix B continued

	Normalised peak area				Relative peak area (%)				
	U70		B70		U70		B70		
	X	S.D.	X	S.D.	X	S.D.	X	S.D.	R (%)
MONOTERPENE HYDROCARBONS	472	92.9	70.7	9.59	25.6	5.63	2.69	0.62	15
FLORAL FRACTION	672	99.6	937	192	36.0	1.35	34.6	1.18	140
SESQUITERPENE HYDROCARBONS	81.7	25.2	34.4	8.92	4.31	0.90	1.26	0.11	42
SPICY FRACTION	643	157	1671	376	34.0	4.26	61.5	1.70	260
TOTAL HOP OIL-DERIVED COMPOUNDS	1868	291	2714	576	100	0.00	100	0.00	145

Appendix C. Composition of the SPE-fraction eluting with 80% EtOH, derived upon SPE fractionation of unboiled (U) and boiled (B) hop essential oil (cv. Saaz).

n°= number of the compound, RI= retention index (RTX-1 capillary column), X= average (n=4), S.D.= standard deviation (n=4), R(%)= recovery of the volatile in B80 vs U80, expressed as %, identification= on the basis of mass spectrum (MS), retention index (RI) and reference compounds (RC). N= only detected in the boiled samples. - = no peak area given (co-elution and peak area negligible).

			Normalised peak area				Relative peak area (%)					
			U80		B80		U80		B80			
n°	**volatile**	**RI**	**X**	**S.D.**	**X**	**S.D.**	**X**	**S.D.**	**X**	**S.D.**	**R (%)**	**Identification**
1	METHYL NONANOATE	1209	0.49	0.25	0.81	0.12	0.01	0.00	0.03	0.01	165	RC, MS, RI
2	ETHYL NONANOATE	1245	5.43	0.84	11.8	1.58	0.10	0.02	0.38	0.05	217	RC, MS, RI
3	METHYL 4,6-DIMETHYLOCTANOATE	1263	1.53	0.34	2.33	0.26	0.03	0.01	0.07	0.01	152	MS
4	UNIDENTIFIED ESTER (m/z 74, 87, 143)	1271	1.79	0.31	3.13	0.31	0.03	0.01	0.10	0.01	175	MS
5	UNIDENTIFIED ESTER (m/z 88, 101)	1280	1.06	0.24	2.26	0.38	0.02	0.01	0.07	0.01	213	MS, RI
6	METHYL *trans*-4-DECENOATE	1289	26.9	6.25	32.9	1.94	0.48	0.15	1.06	0.15	122	MS, RI
7	METHYL *cis*-4-DECENOATE	1299	0.33	0.06	0.42	0.06	0.01	0.00	0.01	0.00	128	MS, RI
8	METHYL DECANOATE	1307	1.74	0.38	3.75	0.65	0.03	0.01	0.12	0.02	216	RC, MS, RI
9	ETHYL *cis*-4-DECENOATE	1361	0.00	0.00	1.19	0.22	0.00	0.00	0.04	0.01	N	MS, RI
10	UNKNOWN METHYL ESTER	1456	0.00	0.00	22.4	22.9	0.00	0.00	0.67	0.61	N	MS
11	METHYL 3,9-DODECADIENOATE	1485	43.2	2.34	58.8	7.21	0.76	0.11	1.88	0.11	136	MS, RI
	TOTAL ESTERS		**82.4**	**10.2**	**140**	**27.8**	**1.46**	**0.30**	**4.44**	**0.44**	**170**	
12	NONANAL	1086	0.83	0.10	0.00	0.00	0.01	0.00	0.00	0.00	0	RC, MS, RI
13	DECANAL	1189	0.20	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0	RC, MS, RI
14	UNIDENTIFIED METHYL KETONE	1238	1.31	0.45	1.71	0.12	0.02	0.01	0.06	0.01	131	MS
15	2-UNDECANONE	1272	23.8	5.35	34.6	1.94	0.42	0.13	1.12	0.15	146	RC, MS, RI
16	2-DODECANONE	1375	28.5	3.12	39.1	4.72	0.50	0.08	1.25	0.13	137	RC, MS, RI
17	2-TRIDECANONE	1475	111	6.86	318	40.9	1.96	0.30	10.2	0.88	286	RC, MS, RI
18	UNKNOWN METHYL KETONE	1574	22.7	1.45	69.3	9.82	0.40	0.05	2.21	0.19	306	MS
19	6Z-PENTADECEN-2-ONE	1644	27.5	2.14	72.8	13.6	0.48	0.05	2.32	0.24	265	MS, RI
20	2-PENTADECAN-ONE	1674	9.72	0.78	25.6	3.24	0.17	0.03	0.82	0.08	263	MS, RI
	TOTAL ALIPHATIC CARBONYL COMPOUNDS		**226**	**15.3**	**561**	**67.9**	**3.99**	**0.61**	**17.9**	**1.32**	**249**	
21	6Z,Z9-PENTADECADIEN-1-OL	1649	9.63	2.50	17.8	3.64	0.17	0.06	0.56	0.07	185	MS, RI
	TOTAL ALIPHATIC ALCOHOLS		**9.63**	**2.50**	**17.8**	**3.64**	**0.17**	**0.06**	**0.56**	**0.07**	**185**	
22	α-PINENE	<1000	1.20	0.18	0.74	0.10	0.02	0.00	0.02	0.00	62	RC, MS, RI
23	CAMPHENE	<1000	0.26	0.03	0.35	0.05	0.00	0.00	0.01	0.00	132	RC, MS, RI
24	β-PINENE	<1000	15.2	1.19	4.02	0.46	0.27	0.03	0.13	0.01	26	RC, MS, RI
25	β-MYRCENE	<1000	861	233	191	31.2	14.9	2.98	6.08	0.43	22	RC, MS, RI
26	P-CYMENE	1013	0.00	0.00	0.59	0.16	0.00	0.00	0.02	0.01	N	RC, MS, RI

Appendix C continued

			Normalised peak area				Relative peak area (%)					
			U80		B80		U80		B80			
n°	volatile	RI	X	S.D.	X	S.D.	X	S.D.	X	S.D.	R (%)	Identification
27	LIMONENE	1021	9.55	1.56	9.05	1.30	0.17	0.01	0.29	0.05	95	RC, MS, RI
28	*Cis*-β-OCIMENE	1027	0.52	0.17	0.51	0.14	0.01	0.00	0.02	0.01	98	RC, MS, RI
29	*Trans*-β-OCIMENE	1038	1.70	0.70	0.33	0.08	0.03	0.01	0.01	0.00	20	RC, MS, RI
30	γ-TERPINENE	1049	0.53	0.08	0.19	0.06	0.01	0.00	0.01	0.00	35	RC, MS, RI
31	TERPINOLENE	1079	0.68	0.11	2.63	0.50	0.01	0.00	0.08	0.01	387	RC, MS, RI
	TOTAL MONOTERPENE HYDROCARBONS		**891**	**236**	**210**	**32.6**	**15.4**	**3.00**	**6.67**	**0.45**	**24**	
32	UNKNOWN MONOTERPENOID (m/z 69, 93, 109, 123, 137, 152)	1073	0.11	0.02	0.26	0.04	0.00	0.00	0.01	0.00	248	MS
33	FENCHYL ETHYL ETHER	1106	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	N	MS, RI
34	LINALYL ETHYL ETHER	1164	0.00	0.00	4.20	0.27	0.00	0.00	0.13	0.01	N	MS, RI
35	TERPINYL ETHYL ETER (ISOMER)	1245	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	N	MS
36	TERPINYL ETHYL ETHER	1254	0.00	0.00	2.66	0.23	0.00	0.00	0.09	0.01	N	MS, RI
	TOTAL OXYGENATED MONOTERPENOIDS AND DERIVATIVES		**0.11**	**0.02**	**7.12**	**0.38**	**0.00**	**0.00**	**0.23**	**0.01**	**6742**	
37	UNKNOWN SESQUITERPENE HYDROCARBON (m/z 91, 105, 119, 147, 175, 190)	1318	0.00	0.00	0.76	0.30	0.00	0.00	0.02	0.01	N	MS
38	(1R,8R,9S)-5,8-CYCLOCARYOPHYLL-4-ENE	1357	0.00	0.00	0.55	0.11	0.00	0.00	0.02	0.00	N	MS, RI
39	α-YLANGENE	1363	2.21	0.45	2.80	0.54	0.04	0.01	0.09	0.02	126	MS, RI
40	α-COPAENE	1368	6.19	1.04	6.31	1.53	0.11	0.03	0.20	0.06	102	RC, MS, RI
41	ISOCARYOPHYLLENE	1393	5.88	1.07	6.42	1.00	0.10	0.03	0.21	0.04	109	MS, RI
42	UNKNOWN SESQUITERPENE HYDROCARBON	1401	1.80	0.44	0.00	0.00	0.03	0.01	0.00	0.00	0	MS
43	*Cis*-α-BERGAMOTENE	1404	0.00	0.00	1.37	0.25	0.00	0.00	0.04	0.01	N	MS, ,RI
44	β-CARYOPHYLLENE	1411	623	41.5	138	19.5	10.9	0.65	4.43	0.74	22	RC, MS, RI
45	CARYOPHYLLA-4(12),8(13)-DIENE	1414	0.00	0.00	3.09	3.56	0.00	0.00	0.09	0.10	N	MS, RI
46	β-COPAENE	1416	12.7	2.21	6.77	4.68	0.23	0.06	0.21	0.12	53	MS, RI
47	*Trans*-α-BERGAMOTENE	1428	38.4	5.11	30.7	6.79	0.68	0.14	0.99	0.26	80	MS, RI
48	α-HUMULENE / β-FARNESENE	1451	3275	373	987	97.9	57.2	1.50	31.7	3.07	30	RC, MS, RI/RC, MS, RI
49	9-EPI-CARYOPHYLLENE	1456	9.50	2.05	0.00	0.00	0.17	0.05	0.00	0.00	0	MS, RI
50	*Trans*-CADINA-1(6),4-DIENE	1461	4.10	0.90	0.00	0.00	0.07	0.01	0.00	0.00	0	MS, RI
51	γ-MUUROLENE	1464	33.1	5.69	39.7	5.94	0.59	0.16	1.28	0.23	120	MS, RI
52	α-AMORPHENE	1466	6.01	0.45	8.11	2.91	0.11	0.01	0.26	0.08	135	MS, RI
53	β-SELINENE	1473	16.6	3.35	15.5	3.41	0.30	0.09	0.50	0.12	93	MS, RI
54	*Cis*-CADINA-1,4-DIENE	1479	21.4	0.44	0.00	0.00	0.38	0.04	0.00	0.00	0	MS, RI
55	α-SELINENE	1482	12.4	1.31	0.00	0.00	0.22	0.04	0.00	0.00	0	MS, RI
56	(Z)-γ-BISABOLENE	1493	4.93	1.06	0.00	0.00	0.09	0.01	0.00	0.00	0	MS, RI
57	β-BISABOLENE	1496	3.70	0.70	0.00	0.00	0.07	0.02	0.00	0.00	0	MS, RI

Appendix C continued

			Normalised peak area				Relative peak area (%)					
			U80		B80		U80		B80			
n°	volatile	RI	X	S.D.	X	S.D.	X	S.D.	X	S.D.	R (%)	Identification
58	γ-CADINENE	1498	23.1	3.57	37.4	8.04	0.41	0.10	1.21	0.31	162	MS, RI
59	*Trans*-CALAMENENE	1502	20.8	2.84	46.9	7.13	0.37	0.08	1.51	0.30	225	MS, RI
60	δ-CADINENE	1507	41.1	4.11	26.1	1.55	0.73	0.14	0.84	0.10	63	MS, RI
61	*Trans*-CADINA-1,4-DIENE	1515	4.06	0.40	0.00	0.00	0.07	0.01	0.00	0.00	0	MS, RI
62	α-CALACORENE	1518	24.8	2.50	36.8	4.52	0.44	0.04	1.18	0.04	148	MS, RI
63	SELINA-3,7(11)-DIENE	1525	2.16	0.37	0.00	0.00	0.04	0.01	0.00	0.00	0	MS, RI
64	α-COROCALENE	1591	1.26	0.22	4.35	0.57	0.02	0.00	0.14	0.01	346	MS, RI
65	CADALENE	1641	3.08	0.54	13.9	2.78	0.05	0.01	0.44	0.07	452	MS, RI
	TOTAL SESQUITERPENE HYDROCARBONS		**4198**	**387**	**1413**	**111**	**73.4**	**1.71**	**45.3**	**3.69**	**34**	
66	UNKNOWN OXYGENTATED SESQUITERPENOID (m/z 79, 93, 107, 121, 135, 145, 163, 205, 220)	1391	1.01	0.16	8.13	0.86	0.02	0.00	0.26	0.03	805	MS
67	4α,8α-EPOXY-CARYOPHYLLANE	1402	0.00	0.00	1.16	0.19	0.00	0.00	0.04	0.00	N	MS, RI
68	UNKNOWN OXYGENATED SESQUITERPENOID (m/z 137, 205, 220)	1428	0.00	0.00	5.70	2.94	0.00	0.00	0.18	0.07	N	MS
69	1,5,8,8-TETRAMETHYL-12-OXA-5-TRICYCLO[7.2.1.0^{6,9}]DODECENE	1462	0.00	0.00	18.7	2.80	0.00	0.00	0.60	0.11	N	MS, RI
70	$\Delta^{2,3}$-5α,8α-EPOXY-CARYOPHYLLANE	1491	0.00	0.00	6.16	1.84	0.00	0.00	0.19	0.04	N	MS, RI
71	4S-DIHYRO-CARYOPHYLLENE-5-ONE / 6(5→4)-ABEO-8,12-CYCLO-CARYOPHYLLAN-5-AL	1524	0.00	0.00	11.8	1.35	0.00	0.00	0.38	0.05	N	MS, RI/MS, RI
72	6(5→4)-ABEO-CARYOPHYLL-7-EN-5-AL	1532	0.00	0.00	1.27	0.48	0.00	0.00	0.04	0.01	N	MS, RI
73	UNKNOWN OXYGENATED SESQUITERPENPOID (m/z 93, 107, 121, 205, 220)	1536	7.49	1.16	17.7	2.79	0.13	0.03	0.56	0.06	236	MS
74	UNKNOWN OXYGENATED SESQUITERPENOID	1541	1.12	0.27	8.73	1.53	0.02	0.01	0.28	0.05	778	MS
75	6(5→4)-ABEO-CARYOPHYLLAN-8(13)-EN-5-AL	1551	3.93	2.21	14.5	3.24	0.07	0.05	0.46	0.07	369	MS, RI
76	CARYOPHYLLENE OXIDE	1554	12.5	3.27	13.0	2.85	0.22	0.08	0.41	0.05	104	RC, MS, RI
77	GLEENOL	1562	0.67	0.41	2.04	0.09	0.01	0.01	0.07	0.01	303	MS, RI
78	HUMULENE EPOXIDE I	1569	16.2	8.68	38.2	6.88	0.30	0.19	1.22	0.17	236	MS, RI
79	HUMULENE EPOXIDE II	1580	36.7	11.2	0.00	0.00	0.66	0.26	0.00	0.00	0	MS, RI
80	4,8,11,11-TETRAMETHYL-8-TRICYCLO-[7.2.0.0^{2,5}]UNDECEN-4-OL	1579	0.00	0.00	18.9	3.01	0.00	0.00	0.60	0.07	N	MS
81	HUMULENE ALLYLIC ALCOHOL	1587	1.03	0.37	5.51	0.98	0.02	0.01	0.18	0.03	535	MS, RI
82	1,10-DI-EPI-CUBENOL	1590	1.32	0.14	4.62	1.18	0.02	0.00	0.15	0.02	350	MS, RI
83	UNKNOWN OXYGENATED SESQUITERPENOID (m/z 79, 93 ,105, 119, 220)	1597	0.00	0.00	20.4	4.03	0.00	0.00	0.65	0.10	N	MS
84	HUMULENE EPOXIDE III	1600	10.3	4.42	26.2	5.23	0.19	0.10	0.83	0.08	255	MS, RI
85	HUMULENOL II	1603	13.6	7.48	14.1	1.61	0.25	0.16	0.45	0.05	104	MS, RI
86	CARYOPHYLLA-4(12),8(13)-DIENE-5α/β-OL	1608	2.81	0.36	5.15	1.10	0.05	0.01	0.16	0.02	183	MS, RI
87	UNKNOWN OXYGENATED SESQUITERPENOID (m/z 67, 81, 95, 107, 133, 159, 187, 202, 248)	1610	0.00	0.00	15.4	2.27	0.00	0.00	0.49	0.06	N	MS

Appendix C continued

			Normalised peak area				Relative peak area (%)					
			U80		B80		U80		B80			
n°	volatile	RI	X	S.D.	X	S.D.	X	S.D.	X	S.D.	R (%)	Identification
88	τ-CADINOL	1612	3.33	0.47	5.15	1.56	0.06	0.01	0.16	0.03	155	MS, RI
89	CUBENOL	1617	1.69	0.22	2.52	0.64	0.03	0.01	0.08	0.02	149	MS, RI
90	3Z-CARYOPHYLLA-3,8(13)-DIENE-5α-OL	1627	4.67	3.76	3.04	0.65	0.09	0.08	0.10	0.01	65	MS, RI
91	EPI-α-BISABOLOL	1656	2.33	0.39	5.49	2.05	0.04	0.01	0.17	0.05	236	MS, RI
92	α-BISABOLOL	1658	0.00	0.00	2.89	1.29	0.00	0.00	0.09	0.03	N	MS, RI
	TOTAL OXYGENATED SESQUITERPENOIDS		**121**	**41.8**	**276**	**44.1**	**2.18**	**0.96**	**8.81**	**0.78**	**229**	
93	PERILLENE	1088	1.54	0.09	0.99	0.24	0.03	0.00	0.03	0.01	64	MS, RI
94	ROSEFURAN EPOXIDE	1171	0.78	0.13	0.00	0.00	0.01	0.00	0.00	0.00	0	MS, RI
95	E-DENDROLASIN	1552	2.69	0.34	6.72	0.44	0.05	0.01	0.22	0.03	250	MS, RI
	TOTAL PYRANS AND FURANS		**5.00**	**0.32**	**7.71**	**0.61**	**0.09**	**0.01**	**0.25**	**0.03**	**154**	
96	UNKNOWN (m/z 57, 85)	1171	-	-	-	-	-	-	-	-	-	
97	UNKNOWN (m/z 57, 85)	1212	0.43	0.07	0.00	0.00	0.01	0.00	0.00	0.00	0	
98	UNKNOWN (m/z 43, 74, 81, 87, 99,127)	1225	0.00	0.00	1.06	0.24	0.00	0.00	0.03	0.01	N	
99	UNKNOWN	1256	0.88	0.20	0.00	0.00	0.02	0.00	0.00	0.00	0	
100	UNKNOWN (m/z 95, 180)	1268	1.02	0.18	0.92	0.08	0.02	0.00	0.03	0.00	91	
101	UNKNOWN (m/z 85, 150)	1298	0.78	0.11	0.80	0.11	0.01	0.00	0.03	0.00	102	
102	UNKNOWN	1302	0.29	0.10	0.31	0.07	0.01	0.00	0.01	0.00	105	
103	UNKNOWN	1380	2.98	0.47	4.38	1.07	0.05	0.01	0.14	0.03	147	
104	UNKNOWN	1387	0.00	0.00	0.88	0.31	0.00	0.00	0.03	0.01	N	
105	UNKNOWN (m/z 79, 80, 81, 93, 122, 136, 164)	1391	1.01	0.16	8.13	0.86	0.02	0.00	0.26	0.03	805	
106	UNKNOWN (m/z 43, 55, 67, 81, 96, 110, 125, 138, 178)	1446	0.00	0.00	21.6	9.50	0.00	0.00	0.67	0.24	N	
107	UNKNOWN (m/z 43, 55, 67, 81, 96, 110, 125, 138, 178)	1453	0.00	0.00	5.15	3.92	0.00	0.00	0.16	0.10	N	
108	UNKNOWN	1467	0.00	0.00	3.11	1.62	0.00	0.00	0.10	0.04	N	
109	UNKNOWN	1479	0.00	0.00	27.8	4.61	0.00	0.00	0.89	0.12	N	
110	UNKNOWN	1515	0.00	0.00	12.2	1.59	0.00	0.00	0.39	0.03	N	
111	UNKNOWN (m/z 79, 80, 81, 150, 157, 220)	1536	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	N	
112	UNKNOWN (m/z 43, 54, 67, 82, 96, 110, 124, 152)	1544	22.9	2.56	74.8	11.5	0.40	0.08	2.39	0.21	327	
113	UNKNOWN	1547	3.38	0.66	0.00	0.00	0.06	0.02	0.00	0.00	0	
114	UNKNOWN (m/z 69)	1584	1.66	0.22	4.66	1.04	0.03	0.01	0.15	0.03	280	
115	UNKNOWN	1608	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	N	
116	UNKNOWN	1616	0.00	0.00	1.85	0.66	0.00	0.00	0.06	0.01	N	
117	UNKNOWN (m/z 80, 93, 112, 121, 136)	1626	0.00	0.00	6.31	1.95	0.00	0.00	0.20	0.06	N	
118	UNKNOWN (m/z 79, 80, 81, 164, 222)	1633	146	13.4	298	61.8	2.56	0.32	9.46	1.29	204	
119	UNKNOWN	1638	5.05	0.60	11.3	2.41	0.09	0.01	0.36	0.04	224	

Appendix C continued

			Normalised peak area				Relative peak area (%)					
			U80		B80		U80		B80			
n°	volatile	RI	X	S.D.	X	S.D.	X	S.D.	X	S.D.	R (%)	Identification
120	UNKNOWN	1652	3.83	0.47	9.46	1.52	0.07	0.01	0.30	0.02	247	
121	UNKNOWN (m/z 82, 85, 109, 121, 139)	1661	0.00	0.00	3.62	1.70	0.00	0.00	0.11	0.05	N	
	TOTAL UNKNOWN VOLATILES		**190**	**16.4**	**496**	**94.7**	**3.34**	**0.45**	**15.8**	**1.61**	**261**	
	MONOTERPENE HYDROCARBONS		**891**	**236**	**210**	**32.6**	**15.4**	**3.00**	**6.67**	**0.45**	**24**	
	FLORAL FRACTION		**104**	**17.8**	**159**	**11.2**	**1.84**	**0.45**	**5.10**	**0.53**	**153**	
	SESQUITERPENE HYDROCARBONS		**4198**	**387**	**1413**	**111**	**73.4**	**1.71**	**45.3**	**3.69**	**34**	
	SPICY FRACTION		**530**	**59.6**	**1347**	**224**	**9.39**	**1.94**	**42.9**	**3.42**	**254**	
	TOTAL HOP OIL-DERIVED COMPOUNDS		**5722**	**529**	**3128**	**317**	**100**	**0.00**	**100**	**0.00**	**55**	

Appendix D. Tentative identification and recoveries (%) of volatiles detected in samples from wort boiling of brew W.

RI= retention index (calculated on RTX-1 column). Area %=relative composition of the sample, based on peak areas. W_{start}= sample taken right after hopping (duplicate analysis; a and b). W_{end}= sample taken after 20 minutes, at the end of the whirlpool process (duplicate analysis; a and b). R(%)= recovery based on full scan normalised peak areas (sample end whirlpool compared to samples start whirlpool). Identification based on MS (mass spectrum), RI (retention index), RC (reference compound) or mixtures of reference compounds (IEP/CEP/HEP= *iso*-caryophyllene/caryophyllene/humulene epoxide, CAA/HAA= caryophyllene/humulene allylic alcohol, HHP/CHP= humulene/caryophyllene hydrolysis product, see section 2.2.1.2). Bold= increase in level of the volatile upon the whirlpool process.

Compound	RI	W_{start} a Area %	W_{start} b Area %	W_{end} a Area %	W_{end} b Area %	R (%)	Identification
α-Pinene	<1000	0.03	0.03	0.03	0.02	38 ± 5	MS/RI/RC
6-Methyl-5-hepten-2-one	<1000	0.12	0.14	0.21	0.22	91 ± 2	MS/RI/RC
β-Pinene	<1000	0.55	0.53	0.40	0.36	39 ± 0	MS/RI/RC
β-Myrcene	<1000	13.0	12.08	8.99	7.79	37 ± 0	MS/RI
α-Phellandrene	<1000	0.06	0.06	0.07	0.07	62 ± 5	MS/RI
Unknown (m/z 55, 82, 110, 111, 127, 142)	1007	1.17	1.25	2.06	1.96	92 ± 3	
Phenylacetaldehyde	1015	0.10	0.12	0.19	0.20	98 ± 0	MS/RI
Limonene	1020	0.33	0.31	0.41	0.38	68 ± 1	MS/RI/RC
Cis-β-ocimene	1026	0.08	0.08	0.14	0.13	92 ± 5	MS/RI/RC
Cis-dihydro-ocimene	1034	0.08	0.08	0.10	0.08	64 ± 3	MS/RI
Trans-β-ocimene	1037	0.19	0.19	0.28	0.26	77 ± 0	MS/RI/RC
Methyl 2-methylheptanoate	1047	0.13	0.13	0.21	0.19	86 ± 6	MS/RI
2-Nonanol	1051	0.00	0.00	0.00	0.00	105 ± 26	MS/RI
Unknown monoterpenoid (67, 71, 79, 81, 93, 107, 122)	**1060**	0.03	0.03	0.07	0.06	**123 ± 8**	
2-Nonanone	1070	1.12	1.16	2.01	1.88	94 ± 2	MS/RI/RC
Terpinolene	1078	0.05	0.05	0.08	0.08	86 ± 2	MS/RI/RC
Linalool	1084	3.02	3.34	5.96	5.55	100 ± 7	MS/RI/RC
Perillene	1086	0.64	0.69	1.06	1.04	87 ± 1	MS/RI
Unknown monoterpenoid (m/z 67, 71, 79, 81, 109, 123, 137, 152)	1097	0.07	0.08	0.10	0.09	74 ± 7	
Myrcenol	**1102**	0.02	0.02	0.07	0.08	**188 ± 6**	MS/RI
Methyl octanoate	1108	0.10	0.11	0.16	0.15	85 ± 2	MS/RI/RC
Unknown monoterpenoid (m/z 69, 79, 91, 107, 121, 152)	1118	0.09	0.09	0.12	0.11	70 ± 3	
Unknown (m/z 67, 69, 71, 79, 91, 137, 156)	1137	0.07	0.09	0.11	0.12	81 ± 0	
Unnown (m/z 69, 79, 91, 107, 121, 150)	**1142**	0.02	0.02	0.03	0.04	**112 ± 1**	
Borneol	**1146**	0.00	0.01	0.01	0.01	**106 ± 1**	MS/RI
Unknown (m/z 43, 54, 67, 81, 96, 111, 125, 136, 154)	**1156**	0.14	0.16	0.32	0.32	**114 ± 4**	
Terpinen-4-ol	1159	0.07	0.08	0.13	0.14	100 ± 8	MS/RI/RC

Appendix D continued

Compound	RI	W start a Area %	W start b Area %	W end a Area %	W end b Area %	R (%)			Identification
α-Terpineol	**1171**	0.14	0.15	0.33	0.34	**126**	±	**2**	MS/RI
2-Decanone	1171	1.37	1.45	2.55	2.56	100	±	2	MS/RI/RC
Ethyl octanoate	1181	0.00	0.00	0.00	0.00	80	±	6	MS/RI
Unknown (m/z 85)	**1183**	0.00	0.00	0.01	0.01	**109**	±	**3**	
Decanal	1184	0.10	0.14	0.20	0.20	90	±	14	MS/RI/RC
2-Decanol	1187	0.30	0.32	0.58	0.56	103	±	4	MS/RI
Methyl 3-nonenoate	1193	0.43	0.44	0.76	0.78	98	±	6	MS/RI/RC
Dodecane	1199	0.02	0.02	0.03	0.04	88	±	26	MS/RI
Unknown (m/z 69, 100)	1202	0.11	0.10	0.16	0.18	88	±	17	
Methyl nonanoate	1207	0.21	0.20	0.28	0.28	73	±	6	MS/RI/RC
Nerol	**1211**	0.01	0.01	0.02	0.02	**135**	±	**1**	MS/RI/RC
Geraniol	**1235**	0.54	0.62	1.19	1.30	**118**	±	**2**	MS/RI/RC
Unidentified methyl ketone	1237	0.83	0.88	1.32	1.29	84	±	0	
Ethyl ester	1245	0.19	0.18	0.19	0.17	53	±	1	
Unknown (unclear mass spectrum)	1252	0.05	0.05	0.08	0.06	75	±	8	
5-Undecen-2-one	1253	1.82	1.94	3.40	3.47	100	±	2	MS/RI
Unknown (m/z 43, 55, 93, 111, 123)	**1257**	0.14	0.15	0.34	0.32	**128**	±	**2**	
Unknown (m/z 69, 114)	1259	0.11	0.12	0.22	0.20	100	±	2	
Methyl ester	1263	0.08	0.07	0.11	0.11	79	±	5	
Unknown (m/z 67, 81, 95, 110)	**1264**	0.19	0.21	0.46	0.42	**121**	±	**13**	
2-Undecanone	1273	4.05	4.07	5.83	5.72	78	±	3	MS/RI/RC
Dihydroedulan	**1278**	0.06	0.06	0.12	0.13	**114**	±	**14**	MS/RI
Vinylguaiacol	**1285**	0.01	0.02	0.04	0.05	**146**	±	**15**	MS/RI
Methyl *trans*-4-decenoate	1289	4.17	4.18	6.19	6.25	82	±	5	MS/RI
Unknown (m/z 85, 150)	1292	1.69	1.81	3.17	3.16	99	±	0	
Unknown (m/z 137)	1295	0.08	0.09	0.15	0.14	98	±	4	
Methyl *cis*-4-decenoate	1299	0.06	0.06	0.08	0.08	72	±	3	MS/RI
Methyl geranate	1301	2.02	2.09	3.45	3.53	93	±	4	MS/RI/RC
Methyl decanoate	1307	0.07	0.07	0.08	0.07	57	±	3	MS/RI/RC
Unknown (m/z 69, 93, 105, 121, 148)	1310	0.06	0.06	0.09	0.10	88	±	12	
α-Cubebene	1342	0.02	0.02	0.01	0.01	28	±	3	MS/RI
Unknown (m/z 43, 54, 68, 82, 96, 124, 161, 189)	1349	0.05	0.05	0.09	0.08	86	±	4	
β-Damascenone	**1358**	0.14	0.15	0.34	0.34	**130**	±	**0**	MS/RI/RC
α-Ylangene	1363	0.08	0.08	0.10	0.09	60	±	3	MS/RI
α-Copaene	1368	0.15	0.13	0.09	0.08	32	±	2	MS/RI/RC
2-Dodecanone	1374	0.44	0.44	0.45	0.45	56	±	2	MS/RI/RC
Unknown (m/z 58, 69, 111, 126) / sesquiterpene hydrocarbon	1378	0.05	0.06	0.07	0.07	70	±	4	

Appendix D continued

Compound	RI	W_{start} a Area %	W_{start} b Area %	W_{end} a Area %	W_{end} b Area %	R (%)		Identification
Unknown (m/z 69, 152, 196)	**1381**	0.22	0.24	0.51	0.50	**122**	± **4**	
Tetradecene	1387	0.17	0.19	0.23	0.22	68	± 5	MS/RI
Unknown (m/z 79, 80, 81, 83, 122, 136, 164)	1390	0.31	0.33	0.45	0.45	77	± 3	
Isocaryophyllene	1395	0.05	0.04	0.03	0.04	44	± 15	MS/RI/RC
Sesquiterpene hydrocarbon (m/z 91, 105, 119, 147, 161, 175, 204)	1402	0.03	0.03	0.03	0.04	55	± 8	
β-Caryophyllene	1407	4.82	4.34	2.46	2.43	29	± 3	MS/RI/RC
Caryophylla-4(12),8(13)-diene	1414	0.02	0.02	0.01	0.01	23	± 1	MS/RI
β-Copaene	1416	0.17	0.15	0.07	0.06	23	± 2	MS/RI
Unknown (m/z 69, 111, 126)	**1418**	0.06	0.06	0.13	0.13	**120**	± **7**	
Trans-α-bergamotene	1425	0.78	0.70	0.45	0.41	32	± 2	MS/RI
Sesquiterpene hydrocarbon (m/z 69, 91, 105, 119)	1430	0.04	0.06	0.06	0.06	65	± 9	
Unknown oxygenated sesquiterpenoid (m/z 69, 81, 95, 109, 123, 138, 149, 177, 191, 205, 220)	1433	0.74	0.92	0.70	0.69	46	± 5	
α-Humulene	1439	21.4	19.8	11.6	12.0	31	± 4	MS/RI/RC
β-Farnesene	1442	8.69	7.44	2.82	2.96	20	± 4	MS/RI/RC
Unknown (m/z 43, 67, 81, 96, 110, 138)	1444	0.31	0.33	0.36	0.37	62	± 2	
Oxygenated sesquiterpenoid (m/z 91, 191, 187, 202)	1450	0.12	0.16	0.14	0.14	53	± 7	
Unknown (m/z 123)	1452	0.07	0.10	0.14	0.14	88	± 16	
β-Ionone	1456	0.33	0.39	0.43	0.50	72	± 3	MS/RI/RC
γ-Muurolene	1460	0.52	0.52	0.34	0.34	36	± 2	MS/RI
α-Amorphene	1463	0.15	0.18	0.16	0.15	50	± 4	MS/RI
Unknown oxygenated sesquiterpenoid (m/z 69, 81, 95, 109, 123, 138, 149, 177, 191, 205, 220)	1468	0.85	1.01	1.21	1.20	71	± 5	
2-Tridecanone	1472	0.93	0.93	0.73	0.79	45	± 4	MS/RI/RC
Cis-cadina-1,4-diene	1477	0.20	0.21	0.19	0.21	52	± 5	MS/RI
α-Selinene	1479	0.29	0.31	0.23	0.22	42	± 1	MS/RI
Epi-zonarene	1482	0.10	0.10	0.08	0.09	46	± 7	MS/RI
Unknown (m/z 79, 80, 81,136) / α-muurolene	1484	0.89	0.97	1.07	1.14	65	± 2	MS/RI
δ-Amorphene	1491	0.04	0.06	0.07	0.08	79	± 6	MS/RI
(E,E)-α-Farnesene	1492	0.06	0.06	0.03	0.04	34	± 6	MS/RI
β-Bisabolene / γ-cadinene	1496	0.80	0.79	0.51	0.51	35	± 3	MS/RI
Trans-calamenene	1500	0.30	0.30	0.26	0.26	47	± 3	MS/RI
δ-Cadinene	1506	0.91	0.86	0.49	0.55	32	± 6	MS/RI
Trans-cadina-1,4-diene	1514	0.15	0.16	0.16	0.17	59	± 5	MS/RI

Appendix D continued

Compound	RI	W_{start} a Area %	W_{start} b Area %	W_{end} a Area %	W_{end} b Area %	R (%)		Identification
α-Calacorene	1517	0.11	0.12	0.12	0.13	58	± 5	MS/RI
4S-Dihydrocaryophyllene-5-one	1523	0.12	0.15	0.19	0.19	77	± 8	MS/RI
6(5-4)-Abeo-caryophyll-7-en-5-al	1532	0.04	0.05	0.07	0.07	81	± 12	MS/RI
Unknown oxygenated sesquiterpenoid (m/z 93, 107, 121, 205, 220)	1534	0.03	0.05	0.07	0.07	94	± 20	
Unknown (m/z 79, 80, 81, 150, 157)	1536	0.23	0.25	0.30	0.31	69	± 0	
E-Nerolidol / caryophylla-4(12),8(13)-dien-5-one	1541	0.10	0.12	0.16	0.15	79	± 9	MS/RI
Caryolan-1-ol	1543	0.01	0.01	0.02	0.02	93	± 1	MS/RI
Humuladienone	1544	0.68	0.79	0.73	0.70	53	± 5	MS/RI
6(5-4)-Abeo-caryophyll-8(13)-en-5-al	1550	0.59	0.73	0.81	0.79	67	± 7	MS/RI
Caryophyllene oxide	1554	0.64	0.70	0.75	0.85	66	± 5	MS/RI/CEP
Clovenol	1555	0.14	0.18	0.24	0.26	85	± 8	MS/RI/CHP
Unknown oxygenated sesquiterpenoid (m/z 107, 135, 218)	1561	0.13	0.15	0.29	0.25	105	± 17	
Humulene epoxide I	1568	1.07	1.28	1.69	1.86	83	± 0	MS/RI/HEP
Humulol	**1574**	0.02	0.02	0.06	0.07	**179**	± **4**	MS/RI/HHP
Humulene epoxide II	1579	2.79	3.20	2.54	2.85	49	± 2	MS/RI/HEP
Humulene allylic alcohol	1586	0.24	0.30	0.39	0.40	80	± 8	MS/RI/HAA
1,10-Di-epi-cubenol	1589	0.18	0.22	0.31	0.33	88	± 3	MS/RI
Junenol / α-corocalene	1591	0.05	0.06	0.08	0.08	86	± 4	MS/RI
Humulene epoxide III	1600	0.59	0.70	0.81	0.86	71	± 1	MS/RI/HEP
Humulenol II	1603	2.57	3.28	3.60	3.66	69	± 7	MS/RI/HAA
Caryohylla-4(12),8(13)-diene-5-ol	1606	0.41	0.50	0.62	0.66	77	± 4	MS/RI/CAA
τ-Cadinol	1612	0.46	0.56	0.87	0.99	101	± 1	MS/RI
Cubenol	1616	0.13	0.16	0.23	0.27	97	± 2	MS/RI
Selin-11-en-4-ol	1621	0.06	0.07	0.10	0.13	100	± 6	MS/RI
α-Cadinol	1624	0.03	0.04	0.06	0.07	101	± 10	MS/RI
3Z-Caryophylla-3,8(13)-diene-5α-ol	1627	0.55	0.67	0.73	0.77	68	± 4	MS/RI/CAA
Unknown (m/z 79, 80, 81, 164, 222)	1631	1.06	1.03	0.81	0.87	44	± 6	
Unknown (m/z 79, 91, 93, 95)	1633	0.37	0.42	0.42	0.50	64	± 5	
Unknkown (m/z 93, 137)	1637	0.08	0.10	0.10	0.11	63	± 0	
3Z-Caryophylla-3,8(13)-diene-5β-ol	1639	0.18	0.23	0.23	0.25	64	± 3	MS/RI/CAA
Unknown (m/z 82)	1644	0.20	0.21	0.16	0.20	48	± 8	
Humulene allylic alcohol	1647	0.40	0.50	0.41	0.44	52	± 4	MS/RI/HAA

LIST OF PUBLICATIONS

PUBLICATIONS IN INTERNATIONAL PEER-REVIEWED JOURNALS:

Praet, T., Van Opstaele, F., De Causmaecker, B., Aerts, G., De Cooman, L. Heat-induced changes in the composition of varietal hop essential oils via wort boiling on lab scale. *Journal of the American Society of Brewing Chemists*, 2016 (*accepted for publication*)

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Dresel, M., Van Opstaele, F., **Praet, T**., Jaskula-Goiris, B., Van Holle, A., Naudts, D., De Keukeleire, D., De Cooman, L., Aerts, G. Evolution des Hopfenaromas von Single Hop-Bieren. *Brauwelt*, 43, 1258-1264, 2014

Dresel, M., **Praet, T.**, Van Opstaele, F., Jaskula-Goiris, B., Van Holle, A., Naudts, D., De Keukeleire, D., De Cooman, L., Aerts, G. Evolution of the aroma of single hop beers. *Brauwelt International*, 110-115, 2015

ORAL PRESENTATIONS:

Praet, T., Van Opstaele, F., Aerts, G., De Cooman, L. The 'kettle hop' aroma mystery: Sesquiterpene oxidation products as character impact compounds. *35th EBC congress*, Porto, Portugal, 2015

Praet, T., Van Opstaele, F., Aerts, G., De Cooman, L. Sesquiterpene oxidation products as key impact compounds for 'kettle hop' aroma. *ASBC annual meeting*, La Quinta (CA), U.S.A., 2015

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Praet, T., Van Opstaele, F., De Causmaecker, B., Aerts, G., De Cooman, L. The journey of hop oil compounds: from kettle to cask. *Proc. Young Scientist Symposium on Malting, Brewing and Distilling (YSS)*, Ghent, Belgium, 2014

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Praet, T., Van Opstaele, F., De Causmaecker, B., Bellaio, G., De Clippeleer, J., De Rouck, G., Aerts, G., De Cooman, L. Hoppy aroma characteristics of conventionally and advanced hopped pilot-scale lager beers. *Proc. 12th Trends In Brewing*, Ghent, 2016

Praet, T., Van Opstaele, F., Aerts, G., De Cooman, L. Heat-induced changes in the composition of varietal hop essential oils via wort boiling experiments on lab scale. *35th EBC congress*, Porto, Portugal, 2015

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